THE ROLE OF THE CYTOSKELETON IN MALIGNANT TRANSFORMATION

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STATEMENT

The study described in this thesis was carried out entirely by the candidate except where indicated in the text and acknowledgements.

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ABBREVIATIONS

ABP	Actin-Binding Protein
cAMP	cyclic AMP
BSA	bovine serum albumin
DMEM	Dulbecco's Modified Eagle's Medium
DNase I	deoxyribonuclease I (EC 3.1.21.1)
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetic acid
EGF	epidermal growth factor
EGTA	ethyleneglycol bis-(aminoethyl)-tetra-acetic acid
FCS	foetal calf serum
cGMP	cyclic GMP
Gu.HCl	guanidine hydrochloride
h	hour
HPLC	high pressure liquid chromatography
MAP	microtubule-associated protein
min	minute
NBD	4-chloro-7-nitrobenz-2-oxa-1,3-diazole
NCS	newborn calf serum
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PMSF	phenylmethylsulphonylfluoride
RP-HPLC	reversed-phase HPLC
RSV	Rous sarcoma virus
s	second
SDS	sodium dodecyl sulphate
SV-40	simian virus 40
TFA	trifluoroacetic acid

ABSTRACT

Malignant cell growth is characterized by uncontrolled proliferation resulting in the formation *in vivo* of progressively growing tumours. The cytoskeleton, through involvement in many cellular functions, may be important in the process of malignant transformation. Of the three major filamentous components of the cytoskeleton, microtubules and intermediate filaments show few alterations associated with the malignant state but the microfilament system of malignant cells is, in general, disorganized. This disorganization is most evident in transformed fibroblasts which lack the dominant stress fibres typical of normal fibroblasts.

Somatic cell hybridization using HeLa/fibroblast cell fusion produces matched pairs of cell lines, one nonmalignant and one a malignant segregant derived from the nonmalignant cell line. The cell lines of each pairing are genotypically very similar but differ in malignant phenotype as assessed by the ability to form progressively growing tumours in nude (athymic) mice. The genotypic similarity of these tumorigenic and nontumorigenic HeLa/fibroblast hybrid cells is a major advantage for an experimental system to be used in the analysis of malignancy.

Microfilament organization in tumorigenic and nontumorigenic HeLa/fibroblast hybrid cells was compared by fluorescence

microscopy. The tumorigenic cells contained fewer, shorter stress fibres, in comparison with the nontumorigenic cells, indicating that the microfilament system of the tumorigenic cells is less organized. In addition, some of the tumorigenic cells contained aggregates of F-actin which may reflect an inability of the tumorigenic cells to form stable F-actin bundles.

The total actin content of the tumorigenic cells was significantly less (by 35%) than that of the nontumorigenic cells while the size of the actin monomer pool was maintained at about 35% of the total actin pool. This suggests that it may be important to cellular function to maintain a pool of actin monomer that is available for rapid polymerization under the influence of certain stimuli. The synthesis of actin, relative to total protein synthesis, was not reduced in the tumorigenic cells and thus could not account for the reduced total actin content of the tumorigenic cells. This decreased actin content is most likely to be due to an increased rate of actin degradation in the tumorigenic cells. Actin monomer is more susceptible to degradation than is actin polymer and actin polymer is in equilibrium with the monomer pool. Instability of polymeric structures in the tumorigenic cells could increase the turnover of actin polymer with actin monomer and thereby increase the availability of actin monomer for degradation. Thus the decreased actin content of the tumorigenic cells is probably a consequence and not the cause of the disorganized microfilament system

observed in the tumorigenic cells.

The organization of actin is regulated by a wide range of actin-binding proteins. The combined activities of these proteins results in a degree of control that allows minor changes in conditions to produce major changes in actin organization. An altered activity of one or more of these proteins could be involved in the disorganization of the microfilament system in tumorigenic cells.

The comparison of fractions, enriched in actin-binding proteins, from tumorigenic and nontumorigenic HeLa/fibroblast hybrid cells did not detect any differences in the protein compositions of these fractions. This result suggests that the expression of actin-binding proteins is not altered with the expression of a tumorigenic phenotype. However, the tumorigenic cell extracts may contain increased amounts of an actin capping/severing activity. An increased amount of this type of activity could be an important factor in the disorganization of the microfilament system and will be an important area for further investigation of the relationship between the microfilament system and the expression of a malignant phenotype.

PUBLICATIONS

GOWING, Linda R., TELLAM, Ross L. & BANYARD, M.R.C. (1984). Microfilament organization and total actin content are decreased in hybrids derived from the fusion of HeLa cells with human fibroblasts. J. CELL SCI. 69:137-146.

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INTRODUCTION

1.1 Malignancy: A Multistage Process

1.1.1 Introduction

Tumour development occurs as a result of continued cell growth in disregard of the normal control mechanisms. Tumours may be benign or malignant, with malignancy being generally defined by properties of invasiveness, metastasis, anaplasia and, usually, rapid growth (Ruddon, 1981). However, distinction between benign and malignant tumours is mostly of clinical significance and in experimental systems like the one utilized for this study, the state of malignancy is more simply defined by the ability of cells to form progressively growing tumours *in vivo*, eventually leading to the death of the host animal.

It is now generally accepted that the development of malignancy *in vivo* is a multistage process (Land *et al.*, 1983; Farber, 1984; Sager, 1984) with stages of initiation and promotion resulting in the growth of a primary tumour (Diamond *et al.*, 1980; Farber, 1984; Weinstein *et al.*, 1984). The study of biochemical changes associated with tumour induction *in vivo* is difficult because of the complexity of the intact animal. The discovery

that nonmalignant cells treated *in vitro* with oncogenic viruses, chemical carcinogens or transfected with tumour cell DNA, acquire phenotypic characteristics typical of malignant cells has enabled the development of *in vitro* models of malignancy. To justify the use of such *in vitro* models it is first necessary to establish that the *in vitro* system is comparable, in certain important aspects, to the *in vivo* situation.

To discuss these *in vitro* systems it is necessary to define terminology as it has been used in the literature and as it will be used here. Cells which have acquired properties characteristic of a malignant phenotype are described as transformed but the state of "transformation" is in many instances assessed only by *in vitro* characteristics. Inoculation of cells grown *in vitro* into suitable test animals makes it possible to assess tumorigenic potential, i.e. the ability of the injected cells to form progressively growing tumours *in vivo* (Harris *et al.*, 1969; Sidebottom, 1980). These tests are usually carried out in immunologically incompetent animals such as newborn mice treated with a sublethal dose of irradiation or athymic (nude) mice, to distinguish between immunologic rejection and failure to grow progressively *in vivo*, i.e. nontumorigenicity, (Harris *et al.*, 1969; Sidebottom, 1980). Through the use of such assays of tumorigenicity, cells maintained *in vitro* can be classified as (1) normal cells which exhibit a finite life span *in vitro*, (2) transformed cells which are nontumorigenic but exhibit an

indefinite life span *in vitro* (they are immortalized or established), and (3) tumorigenic cells which have all the characteristics of transformed cells but, in addition, develop into progressively growing tumours in test animals. The third category of tumorigenic cells can be considered to be malignant since the tumours will eventually cause the death of the host animal.

The expression of at least two separate functions is required for normal cells to acquire a malignant phenotype *in vitro*, (Ruley, 1983): an establishment or transforming function concerned with immortalization of cells and a promotion function leading to full expression of a malignant phenotype. Primary cultures of rodent cells can apparently become tumorigenic in a single step after infection by polyoma virus or adenovirus but these viruses contain at least two genes encoding distinct functions both of which must be expressed for the acquisition of tumorigenicity (Land *et al.*, 1983). NIH 3T3 mouse fibroblasts can, in some cases, be transformed by transfection with a single oncogene but these cells have already acquired characteristics associated with establishment *in vitro* (Land *et al.*, 1983).

While there is no evidence to suggest that the *in vivo* processes of initiation and promotion and the *in vitro* processes of establishment and promotion are equivalent, both processes may contribute to the development of tumorigenicity. Hence it is

likely that any phenotypic characteristics identified as being crucial to the development of tumorigenic potential *in vitro* will also be important to the *in vivo* process.

1.1.2 Characteristics of Transformed and Malignant Cells *In Vitro*

When cells are taken from malignant tumours and maintained *in vitro*, they exhibit a range of phenotypic characteristics not seen in normal cells (Table 1). While no one of these characteristics is an absolute indicator of malignancy, a combination of several characteristics is strongly correlated with the expression of malignancy.

Cells are able to be transformed without becoming tumorigenic. Properties exhibited by transformed but nontumorigenic cells include an indefinite life span, growth to high cell densities and decreased serum requirements for growth. These characteristics, while not intimately associated with malignancy, may promote the acquisition of a malignant phenotype.

Normal cells undergoing division exhibit characteristics such as an increased ratio of nuclear to cytoplasmic volume and increased levels of enzymes involved in the synthesis of nucleic

TABLE 1: Characteristics of transformed and malignant cells maintained *in vitro*.

1. Altered growth properties.
   - *indefinite life span *in vitro*
   - *growth to high cell densities
   - *formation of multilayered colonies
   - *decreased serum requirement
   - *loss of anchorage dependence for growth
   - *ability to grow in semisolid medium
   - *loss of "restriction point" control (i.e. failure to cease growth in G_1 or at the G_1/S boundary of the cell cycle)

2. Alterations of membrane structure and function.
   - *increased agglutinability by plant lectins
   - *alteration in composition of cell surface glycoproteins and glycolipids
   - *loss of cell surface fibronectin
   - *increased uptake of amino acids, hexoses and nucleosides
   - *appearance of tumour-associated antigens
   - *secretion of plasminogen activator
   - *increased membrane fluidity and increased mobility of non-lipid components
   - *increased number of topographical features on upper surface

3. Morphological changes
   - *reduced adhesion
   - *rounded shape
   - *increased number and size of nuclei
   - *loss of cytoskeletal organization

4. Other changes
   - *altered calmodulin levels
   - *reduced cAMP levels or altered cGMP/cAMP ratio
   - *reduced junctional permeability
   - *increased levels of enzymes involved in nucleic acid synthesis
   - *production of growth factors (tumour angiogenesis factor, and sometimes factors causing autocrine growth)
   - *production of oncodevelopmental gene antigens (e.g. oncofoetal antigens, placental hormones or placental-foetal type isoenzymes)

References

Allred & Porter (1979); Hynes (1979); Ruddon (1981); Singer (1982); Azarnia & Loewenstein (1984a,b&c); Veigl *et al.* (1984).

acids. The occurrence of these features in malignant cells reflects a rapid growth rate rather than malignancy *per se*. Levels of cAMP and cGMP have been suggested to be involved in the regulation of the cell cycle, although this remains a controversial subject, and changes in cAMP/cGMP ratios may also reflect the rapid growth rate of malignant cells.

1.1.3 Mechanisms of Transformation

One model of *in vivo* tumour induction that has been extensively studied is the mouse skin model. In this system initiation is a relatively rapid process requiring only a single application of a carcinogen (Diamond *et al.*, 1980). Initiators, or their metabolites, usually bind covalently to cellular DNA (Weinstein *et al.*, 1984) inducing irreversible alterations in the DNA (Diamond *et al.*, 1980; Farber, 1984). In the mouse skin model no apparent morphological changes in the epidermis are associated with initiation (Diamond *et al.*, 1980). In comparison, promotion is a slow and, to some extent, reversible process (Diamond *et al.*, 1980). Promoters, such as the phorbol esters, generally are amphipathic molecules (Diamond *et al.*, 1980) acting primarily at the cell membrane (Weinstein *et al.*, 1984). Promoters possess very weak, if any, carcinogenic activity but markedly increase the

number of tumours formed when applied after a suboptimal dose of an initiating carcinogen (Weinstein *et al.*, 1984).

In view of the diversity of characteristics expressed during the progression towards a malignant phenotype (Table 1) it is important to examine the malignant phenotype at a biochemical level. In a multicellular organism, the regulation of cell proliferation and differentiation in response to the needs of the organism is obviously a complicated process and, as such, is presumably under the control of a variety of genes. Since the basis of tumour development is the disruption of normal growth patterns, it is assumed that the genes activated in malignancy are those normally involved in the regulation of growth and differentiation (Weinberg, 1984; Marshall, 1986). The study of oncogenes, genes whose expression in a cell induces the acquisition of transformed or malignant characteristics, (Marshall, 1986) has supported this assumption.

The first oncogenes to be identified were those of the transforming retroviruses, termed viral oncogenes (v-onc). The finding that these viral oncogenes exhibit homology with normal cellular genes led to the suggestion that the viral oncogenes were derived from normal cellular genes and to the conclusion that the genome of a normal cell contains genes with the potential to become oncogenes. Such normal genes are cellular oncogenes (c-onc) or proto-oncogenes (Weinberg, 1983) and have been

identified in several ways. (1) Through their homology with viral oncogenes of the acutely transforming retroviruses. (2) The chronic transforming retroviruses do not contain a viral oncogene but do contain a viral promoter. Transformation can occur if this viral promoter is inserted adjacent to a proto-oncogene. By determining the point of insertion of the promoter sequence in transformed cells, the proto-oncogene that is transduced can be identified. (3) The active oncogenes in naturally occurring or artificially induced tumours can be detected and isolated using DNA-mediated gene transfer techniques. On occasion, more than one of these strategies has identified the same proto-oncogene.

There is now considerable evidence for viral involvement in some human cancers (Galloway & McDougall, 1983; Tsunokawa *et al.*, 1986; Rowe *et al.*, 1986). The proto-oncogenes that have been identified could, however, be altered to an oncogenic form by agents other than viruses; ultraviolet radiation, chemical carcinogens and tumour promoting agents in the environment and diet are all potentially important in the activation process.

The activation of a proto-oncogene to an oncogenic state may occur by several mechanisms (Hunter, 1984): (1) a simple increase in the concentration of the relevant gene product, (2) the expression of the gene in an inappropriate cell type, (3) unscheduled expression of the gene in a cell type or lineage that normally expresses the gene in a temporally controlled fashion and

(4) alterations in the gene product. Although increased expression of oncogenes occurs in a number of malignant cells, there is little evidence to suggest that abnormal expression of a normal gene is sufficient or even necessary for transformation (Iba *et al.*, 1984; Foster *et al.*, 1985).

It appears that each oncogene, while eliciting several cellular responses, is unable to elicit the full complement of traits required for the expression of tumorigenicity. Synergism among several oncogenes seems to be required. This would be consistent with the multi-step nature of tumour development (Land *et al.*, 1983; Weinberg, 1984). The oncogene(s) activated in a given target tissue may be a function of the activating agent and/or the cell type targeted. Weinberg (1983), in a study of four cell lines independently transformed by a chemical carcinogen, noted that all the transformed cells carried active oncogenes derived from the same precursor proto-oncogene. Given a model of multiple targets in the cellular genome, this result suggests that other proto-oncogenes were in some way less susceptible to activation, or that the carcinogen through a higher binding affinity for certain DNA sequences was selective for one proto-oncogene.

Of the four mechanisms of proto-oncogene activation discussed above, the first three, overproduction, ectopic expression and unscheduled expression, emphasize that defects in the regulation

of gene expression may be important in the development of malignancy. One mechanism that is thought to govern gene expression in eukaryotic cells is that of site-specific DNA methylation which has a strong silencing effect on the expression of genes. Disruption of this methylation may either activate genes or potentiate them for activation. Indeed cancer cells often have disrupted DNA methylation and/or metabolism of methionine, the source of the methyl group, and many carcinogens can disrupt methylation in general with some apparently being specific for DNA methylation (Hoffman, 1984; Kautiainen & Jones, 1986).

More direct evidence for disrupted gene regulation in malignant cells has come from the study of "anti-oncogenes", a term applied by Knudson (1985) to those genes that may regulate the expression of oncogenes. Such genes contribute to cancer in a recessive mode with one normal allele being sufficient to prevent the occurrence of a particular tumour. "Anti-oncogenes" have been implicated in the genesis of human tumours with the most extensively studied examples being retinoblastoma and Wilm's tumour where both alleles of a locus on the short arm of chromosome 13 or 11, respectively, are lost or inactivated. Stanbridge (1985) has extended the concept of anti-oncogenes by suggesting that suppression of the tumorigenic phenotype is not necessarily the result of the anti-oncogene blocking oncogene activation but could also be due to alterations in the target site

of the product of the expressed oncogene. To emphasize this more diverse regulatory mechanism, Stanbridge suggests the term "tumour-suppressor gene" rather than anti-oncogene. This concept of tumour-suppressor genes is supported by experiments in which malignant cells are fused with non-malignant cells. As will be discussed in more detail in the next section, the resulting hybrid cells are, in most cases, non-malignant despite the continued expression of the activated oncogene.

More than 20 proto-oncogenes have now been identified in the human genome and are listed, along with the proposed function and cellular location of the gene product, in table 2. For more detailed information a number of comprehensive reviews have been published (Bishop, 1983; Hunter, 1984; Burgess, 1985; Duesberg, 1985; Hunter, 1985b; Marshall, 1986; Muller, 1986; Weinberg, 1986).

A feature apparent from table 2 is the frequent possession by the oncogene protein products of tyrosine-specific protein kinase activity. Tyrosine-specific kinase activity has also been found to be associated with several growth factor receptors, including those for platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and insulin (Hunter & Cooper, 1983; Nigg *et al.*, 1983; Cochran *et al.*, (1984); Martin *et al.*, 1984; Hunter, 1985a; Hunter & Cooper, 1985). In the normal cell, the binding of a growth factor to the appropriate receptor on the extracellular

TABLE 2: Oncogenes, grouped according to their proposed activity and cellular location.

Gene	Activity	Location of gene product	Comments
Type I: Tyrosine specific protein kinases.			
src	Tyr PK	Plasma membrane (inner face)	cellular homologue $pp60^{c\text{-}src}$
yes	Tyr PK	Plasma membrane (inner face)	
fgr	Tyr PK	?	recombinant of the tyr PK and actin genes
fps	Tyr PK	Plasma membrane and cytoplasm	
fes	Tyr PK	Plasma membrane and cytoplasm	may be the same locus as fps
abl	Tyr PK	Plasma membrane (surface)	
ros	Tyr PK	?	
middle-T	Tyr PK	Plasma membrane (inner face)	from polyoma virus; Tyr PK activity may be due to $pp60^{c\text{-}src}$
Type I related transforming proteins.			
erb-B	Tyr PK	Surface and cytoplasmic membrane glycoprotein	Homology with EGF receptor
fms	?	Surface and cytoplasmic membrane glycoprotein; associated with intermediate filaments	Mimic growth factor activity
raf	?	?	
mil	RNA binding?	cytoplasmic	may be the same locus as raf
mos	?	soluble cytoplasmic	
neu	?	surface	homology with erb-B;

Abbreviations
Tyr PK: tyrosine-specific protein kinase
EGF: epidermal growth factor
PDGF: platelet-derived growth factor

Gene	Activity	Location of gene product	Comments
Type II: GTP binding proteins			
Ha-ras	GTP-binding autokinase	Plasma membrane (inner face)	Normal homologue of Ha-ras and Ki-ras has
Ki-ras	GTP-binding autokinase	Plasma membrane (inner face)	GTP-binding but no autokinase activity;
N-ras	GTP-binding	Plasma membrane (inner face)	isolated from human bladder carcinoma has point mutation
Type III: Growth factor related			
sis	growth factor	secreted and/or cell associated	homology with PDGF
Type IV: Nuclear proteins			
fos	?	nuclear	
myc	DNA binding	nuclear matrix	
myb	?	nuclear	structural homology with myc
B-lym	?	nuclear	product may be related to transferrin
p53	?	nuclear	complexes with SV40 large T antigen and E1b 58K protein of adenovirus
Uncharacterized			
rel	?	?	
erbA	?	cytoplasmic	
ski	?	?	
ets	?	nuclear	

References

Bishop (1983); Smith *et al.* (1983); Hunter (1984); Lane (1984); Naharro *et al.* (1984); Parada *et al.* (1984); Schecter *et al.* (1984); Hunter (1985b); Marshall (1986).

surface of the plasma membrane results in the activation of the tyrosine-specific protein kinase on the cytoplasmic surface of the membrane (Hunter & Cooper, 1983; Hunter *et al.*, 1984; Xu *et al.*, 1984; Hunter & Cooper, 1985). Phosphorylation of the tyrosine residues of specific target proteins may act to transmit the signal from the plasma membrane to the cytoplasm and also to regulate the response of the cell to the receptor-growth factor interaction (Hunter *et al.*, 1984; Hunter & Cooper, 1985). The transforming activity of oncogene products with tyrosine-specific protein kinase activity could be the result of phosphorylation occurring in the absence of growth factor-receptor binding or the phosphorylation of substrates not normally phosphorylated by the growth factor receptor-associated tyrosine-specific protein kinases.

Although some progress has been made in identifying intracellular substrates for these tyrosine-specific protein kinases (Nigg *et al.*, 1983; Martin *et al.*, 1984) in no case has the physiological significance of the phosphorylation been identified (Hunter, 1980) and in many instances the normal function of the potential substrate is unknown (Nigg *et al.*, 1983). In addition there has been recent evidence that the tyrosine-specific protein kinases might also phosphorylate non-protein substrates such as phosphatidylinositol (Hunter & Cooper, 1985).

In normal cells the binding of certain agonists, including neurotransmitters (e.g. acetylcholine) or hormones (e.g. vasopressin) as well as PDGF and EGF, to their appropriate receptors initiates a complex signal cascade (Fig. 1) the key feature of which is the hydrolysis of phosphatidyl inositol 4,5-bisphosphate in the plasma membrane to form diacylglycerol and inositol 1,4,5-trisphosphate. Both diacylglycerol and inositol 1,4,5-trisphosphate have the properties of classical intracellular second messengers in that they are produced very rapidly, they act at low concentrations and there are specific mechanisms for metabolizing these molecules once the external signal is withdrawn (Berridge, 1984). Diacyglycerol activates protein kinase C (the calcium- and phospholipid-dependent protein kinase) in the membrane while inositol 1,4,5-trisphosphate is released into the cytoplasm where it stimulates the release of calcium from intracellular stores, probably the endoplasmic reticulum. The actions of calcium, either directly or via the formation of a complex with calmodulin, and protein kinase C on specific target proteins induce the cellular response, the exact nature of which depends on the type of cell, its differentiation state and the agonist involved (Berridge, 1984; see also Michell, 1983; Nishizuka, 1984; Berridge & Irvine, 1984; Marme & Matzenauer, 1985; Rasmussen et al., 1985).

A number of proteins have been shown to be phosphorylated by protein kinase C but the physiological significance of these

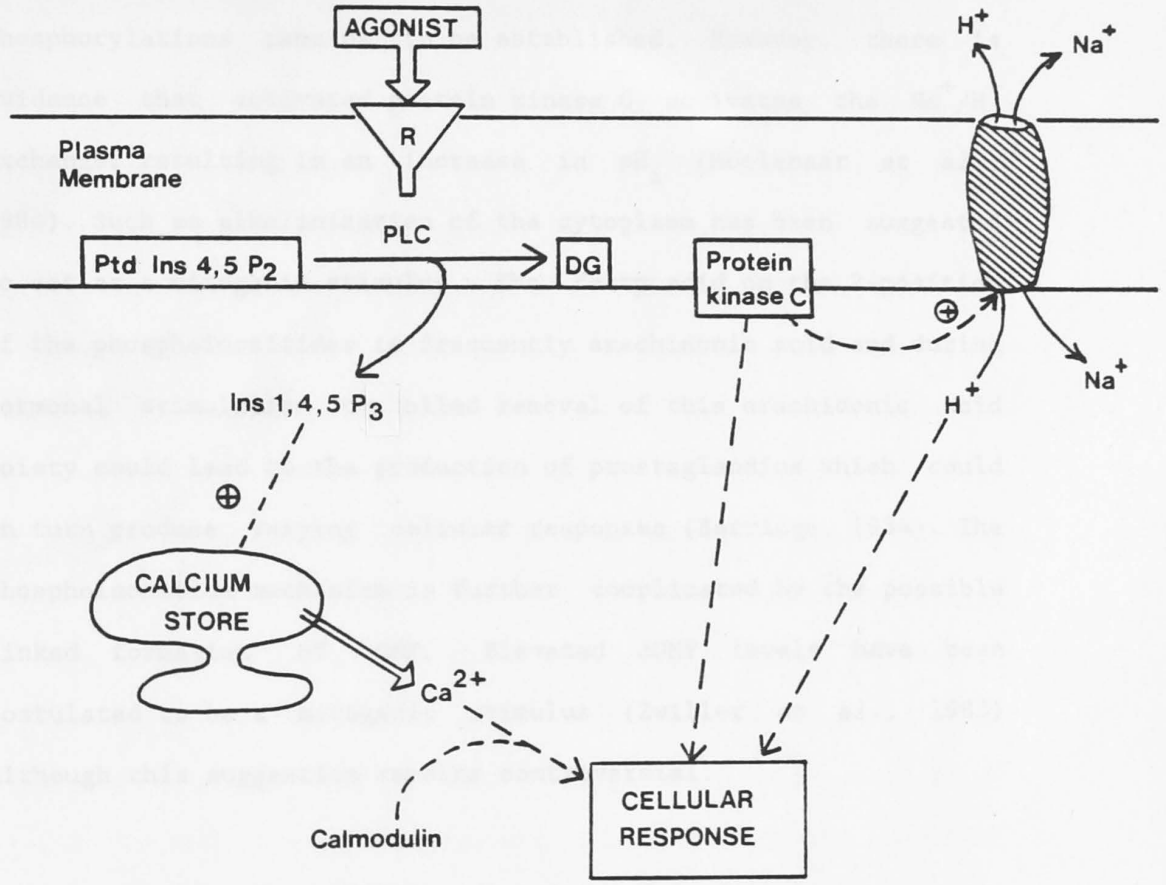


FIGURE 1: Signal transduction by the inositol lipid cascade.

(Adapted from Berridge (1984); see text for details.)

Abbreviations: R = receptor
DG = diacylglycerol
PLC = phospholipase C
PtdIns 4,5 P_2 = phosphatidylinositol 4,5-bisphosphate
Ins 1,4,5 P_3 = inositol 1,4,5-trisphosphate.

phosphorylations remains to be established. However, there is evidence that activated protein kinase C activates the Na^+/H^+ exchanger resulting in an increase in pH_i (Moolenaar *et al.*, 1984). Such an alkalinization of the cytoplasm has been suggested to act as a mitogenic stimulus. The fatty acid on the 2-position of the phosphoinositides is frequently arachidonic acid and during hormonal stimulation controlled removal of this arachidonic acid moiety could lead to the production of prostaglandins which could in turn produce varying cellular responses (Berridge, 1984). The phosphoinositide mechanism is further complicated by the possible linked formation of cGMP. Elevated cGMP levels have been postulated to be a mitogenic stimulus (Zwiller *et al.*, 1985) although this suggestion remains controversial.

A series of enzymatic reactions function to regenerate phosphatidylinositol 4,5-bisphosphate from diacylglycerol and inositol trisphosphate. The ability of tyrosine-specific protein kinases, such as the v-*src* and v-*ros* oncogene protein products, to phosphorylate phosphatidylinositol has led Berridge (1984) to suggest that the normal cellular counterparts of these oncogene products may be kinases which function to channel phosphatidylinositol to the phosphatidylinositol 4,5-bisphosphate used by a cell-surface receptor.

The frequent association of tyrosine-specific protein kinase activity with growth factor receptors and with viral oncogene

protein products suggests that this activity is intimately involved in the regulation of cell growth and maintenance of the transformed state (Hunter & Cooper, 1983). The oncogenic activity of tyrosine-specific protein kinases may be the result of their ability to disrupt the phosphoinositide pathway. Further evidence for disruptions in the phosphoinositide pathway associated with the development of malignancy has come from studies of the tumour-promoting phorbol esters, such as 12-O-tetradecanoyl phorbol-13-acetate (TPA). These compounds seem to act by mimicking the action of diacylglycerol thereby inducing the activation of protein kinase C (Michell, 1983; Kikkawa *et al.*, 1983). However, the activation of protein kinase C by phorbol ester differs significantly from activation by diacylglycerol in that it occurs in the absence of agonist-stimulated inositol lipid breakdown and the resultant increase in intracellular calcium and, in addition, there are no mechanisms to inactivate the phorbol esters.

A number of oncogenes show sequence homology with growth factors or receptors for growth factors (Schecter *et al.*, 1984) and a number of malignant cell lines are known to produce and secrete transforming growth factors similar to the polypeptide growth factors (Twardzik *et al.*, 1982; Maciag, 1983; Burgess, 1985). The oncogene of simian sarcoma virus, v-*sis* shows strong homology with PDGF, the platelet-derived growth factor (Cochran *et al.*, 1984; Hunter, 1985b). The v-*sis* protein has no obvious membrane anchor sequence giving it the potential to be secreted.

It could then presumably bind to the PDGF receptor thereby stimulating continuous growth in an autocrine fashion. The v-*erb-B* oncogene of avian erythroblastosis virus appears to be a truncated form of the EGF (epidermal growth factor) receptor (Downward *et al.*, 1984; Xu *et al.*, 1984), lacking most of the external EGF-binding domain but retaining the proposed membrane anchor domain and cytoplasmic domain (Hunter, 1985b). It is speculated that this oncogene product might imitate an occupied growth factor receptor and thus provide a continuous mitogenic signal in the absence of exogenous growth factors (Hunter, 1985b).

The oncogenes grouped together as Type II in table 2 are all members of the *ras* gene family. Alterations in *ras* genes occur in 10-20% of most human tumour types (Marshall, 1986). The *ras* oncogenes are homologous to a group of normal vertebrate genes (O'Brien *et al.*, 1983) which encode highly related proteins of 189 amino acids designated p21 (Lacal *et al.*, 1984). The p21 *ras* proteins are located on the inner surface of cell membranes, bind GTP and possess a GTPase activity (Der *et al.*, 1986; Walter *et al.*, 1986). This functional activity, together with significant sequence homology to G-proteins (Gilman, 1984) suggests that the p21 *ras* proteins may be analagous to the G-proteins. The known G-proteins are involved in mediating interactions between cell surface receptors and effector enzymes. The effector enzymes may be adenylate cyclase, cGMP phosphodiesterase or possibly diesterases involved in the breakdown of phosphoinositides

(Marshall, 1986). Mutations leading to the substitution of single amino acids at positions 12, 13 and 61 have been identified in human malignancies (Tabin *et al.*, 1982, Lacal *et al.*, 1984; Der *et al.*, 1986). Such substitutions have been postulated to cause conformational changes in the *ras* protein thereby disrupting its function although the loss of GTPase activity does not correlate with transforming activity. Mutations at amino acids 13 and 61 of *ras* have also been identified in human malignancies (Der *et al.*, 1986).

A number of oncogenes (Type IV in Table 2) encode nuclear proteins (Greenberg & Ziff, 1984; Muller & Wagner, 1984; Hunter, 1985a) but there is little known about the functions of these proteins. It has been suggested that the nuclear proteins function as effectors to link events at the cell surface to nuclear events, such as changes in gene transcription and the initiation of DNA synthesis (Marshall, 1986). The c-*myc* gene is expressed early in the G_1 phase of the cell cycle but it is undetectable in resting cells (Hanahan, 1984; Hunter, 1984). In Burkitt's lymphoma a chromosomal translocation causes the *myc* gene to come under the influence of the promoter for antibody production (Klein, 1981) and recently it has been shown that increased expression of the c-*myc* gene confers anchorage-independence and tumorigenic potential on established rat fibroblasts (Pellegrini & Basilico, 1986).

Elevated levels of a protein called p53 occur in a variety of transformed cells (Rotter *et al.*, 1983; Eliyahu *et al.*, 1984; Mercer *et al.*, 1984; Lane & Gannon, 1983; Parada *et al.*, 1984) and substantial amounts are present in actively dividing cells (Mercer *et al.*, 1984; Parada *et al.*, 1984). The p53 proteins may play an important role in the transition of cells from the resting (G_0) to a growing (S) phase but do not seem to be important in cell cycle progression where cells go from G_1 to S phase (Mercer *et al.*, 1984). Introduction of p53 into cells of finite life-span *in vitro* results in cellular immortality and susceptibility to transformation by a *ras* oncogene (Jenkins *et al.*, 1984; Lane, 1984; Parada *et al.*, 1984).

It is apparent from the material presented in this section that if the biochemical basis of malignancy is indeed the disruption of normal growth patterns, such disruption can occur at a number of different levels: (1) growth factors; (2) growth factor receptors; (3) effector systems such as the tyrosine-specific protein kinases, the phosphoinositide pathway or the G-proteins, all of which function to transmit a signal from growth factor-receptor complexes to the cytoplasm of the cell; (4) the nucleus; (5) regulatory mechanisms such as the anti-oncogenes or tumour suppressor genes which may provide control at any or all of the above levels. Since more than one activated oncogene is required for tumorigenicity it seems likely that disruption at more than one level must occur before the malignant phenotype is

expressed.

1.1.4 Somatic Cell Hybrids: A Model System

The development of malignancy *in vivo* and *in vitro* is a multistage process, associated with the acquisition of a range of phenotypic characteristics. A comparison of normal, transformed and tumorigenic (malignant) cells should allow distinction between those phenotypic characteristics that are associated with transformation from those that are linked to the development of tumorigenicity. Any characteristics that are intimately associated with the development of tumorigenicity *in vitro* are likely to also be important to the development of cancer *in vivo*.

The study of transformed, tumorigenic and normal cells *in vitro* has been aided by the development of two types of experimental models. One of these models utilizes transformation-defective, temperature-sensitive mutants of the transforming retroviruses such as Rous sarcoma virus. Cells infected with these mutants express phenotypic characteristics typical of malignant cells at the permissive but not at the restrictive temperature, while viral replication proceeds unaffected at both temperatures (David-Pfeuty & Singer, 1980;

Boschek *et al.*, 1981). However, the usefulness of such systems for study is limited as it is difficult to assess the tumorigenicity of the cells at temperatures removed from the physiological norm, a limitation that does not apply to the second model system, that of somatic cell hybrids. The development of somatic cell hybridization as an experimental technique and its use in studies of gene regulation and expression has been reviewed by Harris (1970) and Stanbridge *et al.* (1982).

The application of somatic cell hybridization to the analysis of malignancy was limited until the development of the Sendai virus cell fusion technique by Harris & Watkins (1965) which made possible the fusion of virtually any mammalian cells. Initial studies in this field (Barski & Cornefert, 1962) were carried out using inter- and intra-specific rodent hybrid cells resulting from the fusion of a highly malignant cell with a nonmalignant cell. The hybrid cells produced in this way were tumorigenic, and thus it was concluded that malignancy behaves as a dominant trait. However, Harris *et al.* (1969) carried out a more detailed study using mouse cells which demonstrated that malignancy can be suppressed when malignant cells are fused with certain non-malignant cells. In addition, the hybrid cells resulting from such fusions produce segregants in which a loss of chromosomes is associated with reversion to malignancy (Harris *et al.*, 1969). A feature of the rodent hybrid cells is their chromosomal instability which results in a significant proportion of the total

chromosomal complement being rapidly lost, leading to a rapid re-expression of malignancy. It was this feature that resulted in the initial misleading conclusion that malignancy is a dominant trait. Studies by Stanbridge *et al.* (1982) and by Klinger (1980) demonstrated that human-human hybrid cells have a considerably more stable chromosome complement, in comparison with the rodent cell system, thus allowing more extensive studies to be carried out.

It is now well established that, when a number of conditions are met, the fusion of malignant cells with normal diploid cells results in hybrid cells which have their tumorigenic capacity suppressed. However, after a period of continued cultivation *in vitro*, tumorigenic segregants can be isolated from the hybrid cell population. This re-expression of tumorigenicity is associated with the loss of chromosomes derived from the diploid parent (Klinger, 1980; Sidebottom, 1980; Stanbridge *et al.*, 1982; Harris, 1986) indicating that tumorigenicity behaves as a recessive trait and that the chromosome(s) contributed by the normal parent must carry determinants that can neutralize the defect(s) responsible for malignant behaviour (Klinger, 1980; Lagarde & Kerbel, 1985; Stanbridge, 1985; Harris, 1986).

It should be emphasized here that the tumorigenicity of somatic cell hybrids such as the HeLa/fibroblast system of Stanbridge *et al.* (1982) and Klinger (1980) is only suppressed,

not corrected by the determinant provided by the chromosomes of the normal parent. The potential for tumorigenicity remains latent until the chromosome(s) containing the gene(s) coding for the suppressive element(s) is (are) lost. This suppression mechanism is also operative *in vivo*. After injection of the hybrid cells into nude (athymic) mice, both tumorigenic and nontumorigenic cell populations divide actively for the first few days post-inoculation, but while the tumorigenic cells then continue to divide, the nontumorigenic cells show a decline in mitotic activity and a shift to a more flattened, fibroblast-like morphology. However, viable cells can at any stage be retrieved by biopsy indicating that the lack of tumour production is due to suppression of mitotic activity and is not the result of natural killer mediated cytotoxicity (Stanbridge & Ceredig, 1981).

The conclusion that the re-expression of tumorigenicity in somatic cell hybrids is associated with the loss of chromosomes derived from the diploid parent, led investigators to question whether a specific chromosome(s) could be identified that carries a gene(s) relevant to the manifestation of tumorigenicity. In the case of mouse/mouse hybrids, the chromosomes 4 derived from the diploid parent cell have a decisive role in suppressing the malignant phenotype and this suppression is subject to gene dosage effects: it is reinforced by an increase in the number of diploid chromosomes 4 in the hybrids and it may be overcome by an increase in the number of chromosomes 4 derived from the malignant parent

cell (Harris, 1986). In the intraspecies human HeLa x diploid fibroblast cell hybrids the loss of one copy of chromosome 11 from the diploid parent, and possibly others, correlates with the re-expression of tumorigenicity (Stanbridge *et al.*, 1982; Stanbridge, 1985; Kaelbling & Klinger, 1986).

The further question as to whether these genes are unique or common to distinct types of cancer was approached by fusion of two malignant cell types. Complementation and suppression of tumorigenicity in the hybrid offspring would be expected if the mechanisms of transformation of the two malignant parents were different. Hybrids produced by crosses of malignant mouse cells were in virtually all instances highly malignant (Wiener *et al.*, 1974) suggesting that the lesions determining the malignant phenotype of a wide range of mouse tumours, although recessive, fail to complement each other (Wiener *et al.*, 1974). However, in making any interpretation of this result the chromosomal instability of these hybrids must be taken into consideration. In the case of human cell lines, carcinoma x carcinoma and carcinoma x lymphoblastoid hybrid cells were found to be tumorigenic while carcinoma x sarcoma and carcinoma x melanoma hybrid cells were nontumorigenic (Weissman & Stanbridge, 1983). This led to the suggestion (Weissman & Stanbridge, 1983) that a family of genes, possibly a distinct one for each somatic cell type, controls the expression of tumorigenicity. A similar suggestion has been made based on the evidence obtained from the study of viral and

cellular oncogenes.

The system of somatic cell hybrids derived from the fusion of the tumorigenic D98/AH-2 clone of HeLa with normal diploid human fibroblasts (Stanbridge *et al.* 1982; Klinger, 1980) has a number of advantages as an experimental model for the study of malignancy. Firstly, Stanbridge *et al.* (1982) observed that immediately following fusion, most hybrid clones had lost less than 5% of chromosomes based on the sum of the chromosomal numbers of the parental population. As a result of this relative chromosomal stability, the degree of chromosomal loss associated with the re-expression of tumorigenicity is minimized and the nontumorigenic hybrid cells are genotypically very similar to the tumorigenic segregants derived from them. Any differences in phenotypic charateristics in the tumorigenic segregants can, therefore, be expected to be related to the expression of tumorigenicity.

Secondly, the hybrid cells have the characteristics of transformed cells (Stanbridge & Wilkinson, 1978; Klinger, 1980) in that they grow rapidly in the presence of low serum, exhibit an indefinite life span in vitro, and reach high population densities similar to those attained by the parental HeLa cells (Stanbridge *et al.*, 1982). This system therefore provides an opportunity to evaluate the specific association of *in vitro* phenotypic markers with the *in vivo* property of tumorigenicity. A marker that is

intimately linked with tumorigenicity must be present in the parent malignant cell but not in the normal parent; it must disappear in the nontumorigenic hybrid cells and must reappear in all independently derived tumorigenic segregants (Atkinson & Bramwell, 1980; Sidebottom, 1980).

Thirdly, the HeLa cell line was derived from a naturally occurring human tumour and therefore, whatever the mechanism leading to malignancy, it should be applicable to human cancer.

Fourth, cell fusion events may occur *in vivo* either spontaneously or as the result of viral activity (Lagarde & Kerbel, 1985). Tumour cell/host cell or tumour cell/tumour cell fusion events may be one of several means by which tumour cell populations diversify genotypically, become heterogeneous and generate highly aggressive malignant variants.

Fifth, the morphology of the hybrid cells is intermediate between that of the epithelial and fibroblastic parents (Stanbridge *et al.*, 1982), thus allowing comparisons to be made without the complexities that can be introduced by large variations in morphology. This is particularly important for a study of the cytoskeleton, the structure of which is closely linked with cell morphology.

Previous studies of somatic cell hybrid systems have shown

that a number of characteristics including an infinite life-span, reduced requirement for serum growth factors, anchorage independent growth, production of plasminogen-activating enzyme and decreased fibronectin production are all expressed by both non-tumorigenic and tumorigenic hybrid cells (Stanbridge & Wilkinson, 1978; Klinger, 1980; Sidebottom, 1980; Der *et al.*, 1981; Stanbridge *et al.*, 1982 & 1983). Hence, although these properties may potentiate the development of tumorigenicity, they are not essential for malignancy *per se* (Klinger, 1980). However, the re-expression of tumorigenicity in these hybrid cell systems is associated with alterations in the expression of activity of a number of membrane proteins (Atkinson & Bramwell, 1980; Sidebottom, 1980; White *et al.*, 1981) and alterations in the distribution of extracellular fibronectin and intracellular microfilaments (Der *et al.*, 1981; Stanbridge *et al.*, 1982).

1.2 The Cytoskeleton: Structure and Function

1.2.1 Introduction

The cytoplasm of eukaryotic cells, far from being a formless broth bathing the organelles, exhibits a high degree of spatial and functional order. It is the cytoskeleton, a dynamic, interacting system of tubules and filaments which provides this capacity for organization so essential for normal cellular functioning (Brinkley, 1981).

The cytoskeleton has been operationally defined as that portion of the cell which maintains its integrity upon extraction with non-ionic detergents in appropriate buffers. However, this definition is limited and does not allow for the dynamic nature of the cytoskeleton. A more encompassing view of the cytoskeleton is as a three-dimensional, filamentous network comprised of a structural component, the protein polymers which form the filaments, and a regulatory component, proteins associated with the filaments which control the organization and interactions of the cytoskeleton.

The cytoskeleton is composed of three major filamentous systems, the microtubules, microfilaments and intermediate filaments, named for their appearance under the electron microscope. Microtubules appear as long, hollow tubular structures with an outer diameter of 25nm, a central lumen of 15nm diameter and lengths ranging from 10 to 25 micrometers. Microfilaments are thin, often branching filaments of 4 to 6nm diameter and intermediate filaments with a diameter of 8 to 11nm are 'intermediate in size' to microfilaments and microtubules. The existence of a fourth filamentous component termed microtrabeculae has been postulated from high voltage electron microscopy (Porter & Tucker, 1981; Schliwa *et al.*, 1982; Ben-Ze'ev, 1985) but recent evidence suggests that the microtrabeculae are artifacts induced by the presence of water or ethanol during the critical point drying procedure used for these studies (Ris, 1985).

The cytoskeleton-associated proteins are defined by their ability to co-isolate and interact *in situ* with one or more of the filamentous networks and may effect dynamic changes of cytoskeletal architecture *in vivo* by (1) altering the process of polymerization of the protein subunits into the filamentous structures, (2) engendering a redistribution of intracellular filaments, (3) serving as potential cross-bridging agents linking the filamentous systems to each other and to other subcellular organelles or (4) via specific catalytic activities (Napolitano *et*

al., 1985).

The general characteristics of microtubules and microfilaments have been extensively reviewed (Snyder & McIntosh, 1976; Goldman *et al.*, 1979; Brinkley, 1981; Weber & Osborn, 1982; David *et al.*, 1983; Porter, 1984; Satir, 1984). The discussion here aims to provide a brief overview of the structure and putative functions of the cytoskeletal components with emphasis on the regulatory mechanisms governing this organization, particularly in non-muscle cells.

1.2.2 Microtubules

1.2.2.1 Structure

Microtubules are polymers of the protein tubulin, a dimer of two closely related globular polypeptides of 50,000 M_r, alpha- and beta-tubulin. Under appropriate conditions, the *in vitro* assembly of microtubules from tubulin dimer will occur (Fig. 2). The first step of the process, nucleation, involves the formation of stable aggregates of tubulin dimers. These 'nuclei' are then elongated by the addition of more dimers to form polymers termed

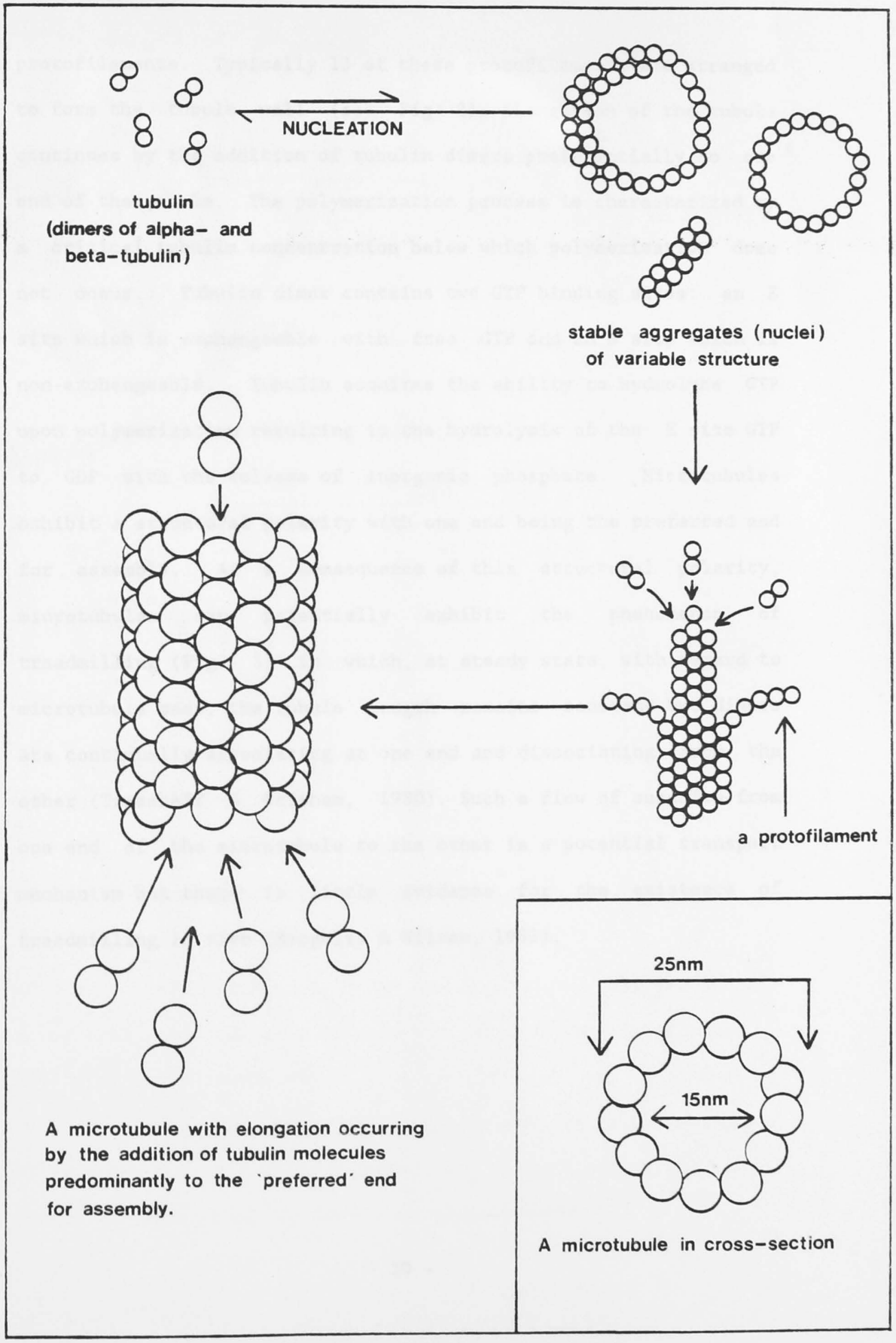


FIGURE 2: Tubulin polymerization and microtubule structure.

protofilaments. Typically 13 of these protofilaments are arranged to form the tubule wall (see Fig. 2). Elongation of the tubule continues by the addition of tubulin dimers preferentially to one end of the tubule. The polymerization process is characterized by a critical tubulin concentration below which polymerization does not occur. Tubulin dimer contains two GTP binding sites: an E site which is exchangeable with free GTP and an N site which is non-exchangeable. Tubulin acquires the ability to hydrolyze GTP upon polymerization resulting in the hydrolysis of the E site GTP to GDP with the release of inorganic phosphate. Microtubules exhibit a structural polarity with one end being the preferred end for assembly. As a consequence of this structural polarity, microtubules can potentially exhibit the phenomenon of treadmilling (Fig. 3) in which, at steady state, with regard to microtubule mass, the tubule length remains constant but dimers are continually associating at one end and dissociating from the other (Timasheff & Grisham, 1980). Such a flow of subunits from one end of the microtubule to the other is a potential transport mechanism but there is little evidence for the existence of treadmilling *in vivo* (Margolis & Wilson, 1981).

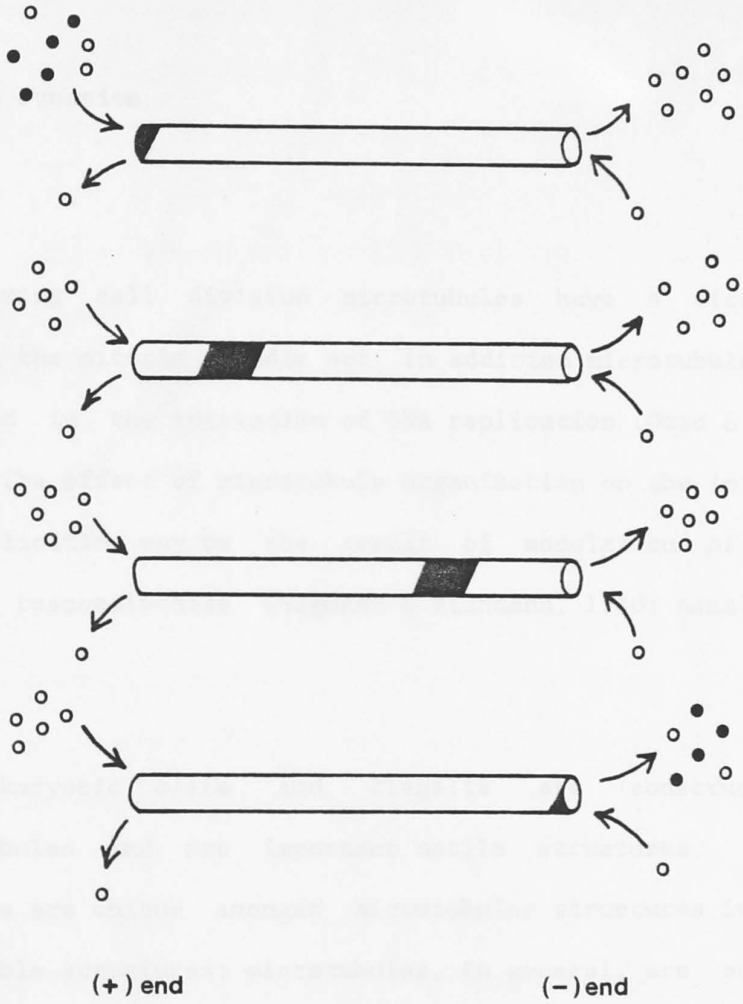


FIGURE 3: Treadmilling of microtubule protomers.

At steady state tubulin protomers are continually adding to and being lost from both ends of the microtubules. In the presence of GTP, protomer addition occurs preferentially at one end (the + end) and dissociation from the opposite end. As a consequence a relative motion of tubulin protomers can occur from one end to the other, as represented by the shaded bands.

1.2.2.2 Function

During cell division microtubules have a vital role in forming the mitotic spindle but, in addition microtubules may be involved in the initiation of DNA replication (Otto & De Asua, 1983). The effect of microtubule organization on the initiation of DNA replication may be the result of modulation of adenylate cyclase responsiveness (Hagmann & Fishmann, 1980; Aszalos *et al.*, 1985).

Eukaryotic cilia and flagella are constructed from microtubules and are important motile structures. Cilia and flagella are unique amongst microtubular structures in that they are stable structures; microtubules, in general, are one of the most labile of the cytoskeletal structures and a dynamic equilibrium between microtubules and its subunits seems to be essential for normal function (Margolis & Wilson, 1981).

Certain blood cells, including mammalian platelets and the nucleated erythrocytes of non-mammals, contain a bundle of microtubules, the marginal band, encircling the peripheral region of the cytoplasm. It is this band of microtubules that maintains the characteristic shape of these cells (Kenney & Linck, 1985). In

platelets, stimulated with collagen, thrombin or platelet-activating factor, the marginal band is rapidly disassembled, probably in response to an induced calcium flux (Feinstein *et al.*, 1985), thereby facilitating the characteristic shape change associated with stimulation. In most cells the nucleus is surrounded by a network of microtubules, presumably providing support to the nucleus as well as helping to maintain the shape of the cell.

Microtubules are involved in intracellular transport activities such as fast axonal transport (Allen *et al.*, 1985; Vale *et al.*, 1985), chromosome separation during mitosis (David *et al.*, 1983), the movement of secretory and pigment granules as well as the various membrane-bound organelles. There is some debate as to the exact role of microtubules in these motile functions. The microtubules have been suggested to act as 'rails' with a contractile system based on microfilaments providing the motile force (David *et al.*, 1983). Alternatively, the motion may be provided by the 'treadmilling' of tubulin subunits along the microtubule (Margolis & Wilson, 1981) or by the interaction of microtubules with a specific protein (Vale *et al.*, 1985). The latter possibility will be discussed further in section 1.2.2.3.

As well as mediating organelle transport, microtubules may be involved in controlling the cytoplasmic distribution of organelles. The mitochondria are closely associated with both

microtubules and intermediate filaments but not with microfilaments (Chen *et al.*, 1984). Microtubules also seem to maintain the stacks of cisternae of the Golgi apparatus in a configuration that ensures a normal flow of material through that system (Thyberg & Moskalewski, 1985). Involvement of microtubules is also indicated in the stabilization of cell-substrate contacts (Geiger *et al.*, 1984), in nerve cell differentiation (Edde *et al.*, 1982; Cumming & Burgoyne, 1983) and in the phenomena of cell surface receptor patching and capping.

1.2.2.3 Microtubule-Associated Proteins

The majority of microtubule-associated proteins are usually grouped into two major classes: the high molecular weight (HMW) or microtubule-associated protein (MAP) fraction and the *tau*-factor proteins (Table 3). All of these proteins promote tubulin polymerization, probably by stabilizing the polymeric structure. MAPs bind very rapidly to binding sites on the tubulin polymers and then exchange from these sites. At very low concentrations (6% of the tubulin concentration), MAPs cause measurable stabilization of local microtubule regions. Job *et al.* (1985) suggest that this local stabilization may cause different stability classes of microtubules. *In vitro* MAP-2 and *tau* can

TABLE 3: Microtubule-associated proteins.

Name	M_r	Comments
MAP-1	300,000	Subdivided into MAP-1A, MAP-1B and MAP-1C; heat sensitive and do not show immunological cross-reactivity with each other or with the MAP-2 class.
MAP-2	300, 000	Subdivided into MAP-2A and MAP-2B; heat stable and immunologically cross-reactive with each other.
MAP-3	180,000 doublet	In adult brain concentrated in axons and glial cells whereas MAP-1 and MAP-2 are concentrated in dendrites.
tau factor	55,000-62,000	Stabilize polymeric tubulin structure
dynein	600,000	ATPase which generates energy for motile action of cilia and flagella
kynesin	600,000	May generate energy for axonal transport and organelle movement.

References

Murphy & Borisy (1975); Sloboda *et al.* (1975); Weingarten *et al.* (1975); Sloboda *et al.* (1976); Scherson *et al.* (1984); Vallee *et al.* (1984); Huber *et al.* (1985); Vale *et al.* (1985); Huber *et al.* (1986).

compete for binding sites on the microtubule surface but *in situ* these proteins may be localized in separate intracellular compartments. In brain at least, *tau* is restricted to neuronal axons while MAP-2 is concentrated in neuronal cell bodies and dendrites (Binder *et al.*, 1985).

The appearance of the microtubule-associated proteins under the electron microscope is as fine filamentous arms on the surface of the microtubules, suggesting a potential role in the mediation of interactions between microtubules and other cellular organelles (Timasheff & Grisham, 1980; Vallee *et al.*, 1984); there is increasing evidence for such a role. After dissolution of the microtubules, MAP-2 has been found, by immunocytochemical techniques, associated with the intermediate filaments (Bloom & Vallee, 1983; Vallee *et al.*, 1984). *In vitro*, microtubules and microfilaments will interact to form a gel, but only in the presence of MAPs (Griffith & Pollard, 1982; Pollard *et al.*, 1984). MAP-2 will crosslink purified actin filaments into bundles (Arakawa & Frieden, 1984; Pollard *et al.*, 1984; Sattilaro, 1986) and purified mammalian brain neurofilaments will form a complex with MAP-1, MAP-2 and tubulin (Leterrier *et al.*, 1982). Such interactions may be regulated by phosphorylation of MAPs (Selden & Pollard, 1983; Pollard *et al.*, 1984; Vallee *et al.*, 1984; Sattilaro, 1986) on the domain which projects from the surface of the microtubule (Bloom & Vallee, 1983) and, potentially, also by intracellular pH changes (Nishida *et al.*, 1981). Microtubule

assembly is influenced by micromolar concentrations of calcium, probably through the interaction of a calcium-calmodulin complex with MAP-2 or *tau* proteins (Kakiuchi *et al.*, 1982; Lee & Wolff, 1984).

Cilia and flagella are constructed from an outer ring of nine doublet microtubules plus two central microtubules. Each of the nine doublets is attached to the central microtubules by radial spokes, and emerging from each doublet are 'arms' which are the protein dynein. In the presence of ATP, the dynein arms form bridges between adjacent microtubule doublets; the breakage of these bridges requires the hydrolysis of ATP. It is thought that via cycles of bridge formation and release, the dynein arms of one doublet walk along the adjacent doublet causing the outer doublets of microtubules to slide past each other. The structure of cilia and flagella is such that this sliding action, driven by the energy of ATP hydrolysis, is then translated into the typical beating action of these structures (Snyder & McIntosh, 1976; Stryer, 1981). Kinesin, a recently characterized microtubule-associated protein, is a potential candidate for the provision of the motile force in fast axonal transport, and possibly in organelle movement (Vale *et al.*, 1985). Although not usually defined as a microtubule-associated protein, clathrin, the major protein of coated pits and vesicles is able to associate reversibly with microtubules (Imhof *et al.*, 1983) suggesting the potential involvement of microtubules in endocytosis and

processing of these structures.

1.2.3 Microfilaments

1.2.3.1 Structure

Microfilaments are formed by the polymerization of a 42,000 M_r globular protein, actin, which is a major constituent of all eukaryotic cells. The primary amino acid sequence of actin has been highly conserved during evolution. Except for a series of variable acidic residues near the amino terminus, there are only minimal (less than 5%) differences in the sequences of muscle and cytoplasmic actins of animals and protozoa (Oosawa, 1983; Pollard & Cooper, 1986). In higher organisms there are multiple actin genes that are differentially expressed in various tissues; mammals, for example, express at least 6 actin genes (Vandekerckhove *et al.*, 1984). The products of the various genes exhibit the same molecular weight but are detectable, as actin isoforms, by differences in isoelectric point (alpha-actin being the most acidic and gamma-actin the most basic isoform). In differentiated vertebrate striated muscle alpha-actin is the dominant isoform while non-muscle cells contain predominantly

beta- and gamma-actins (Clarke & Spudich, 1977; Goldman *et al.*, 1979; Otey *et al.*, 1986). Although no major functional differences have been detected for the different actin isoforms (Goldman *et al.*, 1979; Otey *et al.*, 1986), changes in the ratio of isoforms have been related to the state of cellular proliferation and differentiation (Saborio *et al.*, 1979; McRobbie & Newell, 1985; Strauch *et al.*, 1986). This observation will be discussed further in relation to neoplastic transformation in Section 1.3.2.

The properties of actin and the mechanism of *in vitro* polymerization have been studied in some detail (Pollard *et al.*, 1981; Korn, 1982; Oosawa, 1983; Pantaloni *et al.*, 1985; Wanger *et al.*, 1985; Pollard & Cooper, 1986). The discussion here highlights points which are important and relevant to this thesis.

Under conditions of low ionic strength, actin is in a monomeric state (globular or G-actin). On the addition of neutral salts of physiological ionic strength or millimolar concentrations of divalent cations, G-actin undergoes a process of self-assembly to form long polymers (filamentous or F-actin). The F-actin polymer (a microfilament) is a double-helical structure with 13.5 protomers per turn. More complex structures such as bundles, two- and three-dimensional networks are formed by the association of actin filaments (Fig. 5).

Like tubulin polymerization, actin polymerization is

characterized by a nucleation/elongation mechanism and a critical concentration for polymerization (Fig. 4). Actin in excess of the critical concentration directly becomes actin polymer, but a pool of actin monomer, equal to the critical concentration, is always maintained at steady state with respect to actin polymer.

The rate-limiting step in the kinetics of actin polymerization (Fig. 5) is the nucleation step in which 3 or 4 monomers cooperate to form a stable nucleus. Once the nucleus is formed, rapid elongation occurs by the bidirectional addition of monomers to the polymer ends. End to end annealing of actin polymers can also occur. The presence of nucleotides, especially ATP, is necessary to maintain the native conformation of G-actin. The ability of nucleotides to accelerate polymerization is probably due to the stabilization of G-actin in a conformation favourable for polymerization. G-actin binds one molecule of ATP and during the transition to actin polymer ATP hydrolysis occurs but it is not essential for polymerization as ADP-actin can also polymerize, albeit at a slower rate (Pollard, 1984). However, like the GTP hydrolysis associated with tubulin polymerization, the ATP hydrolysis has a considerable effect on the kinetics of actin polymerization and on the stability of the resultant polymers. Divalent cations bound to specific sites on G-actin also stabilize the native conformation of G-actin and thereby regulate the polymerization rate.

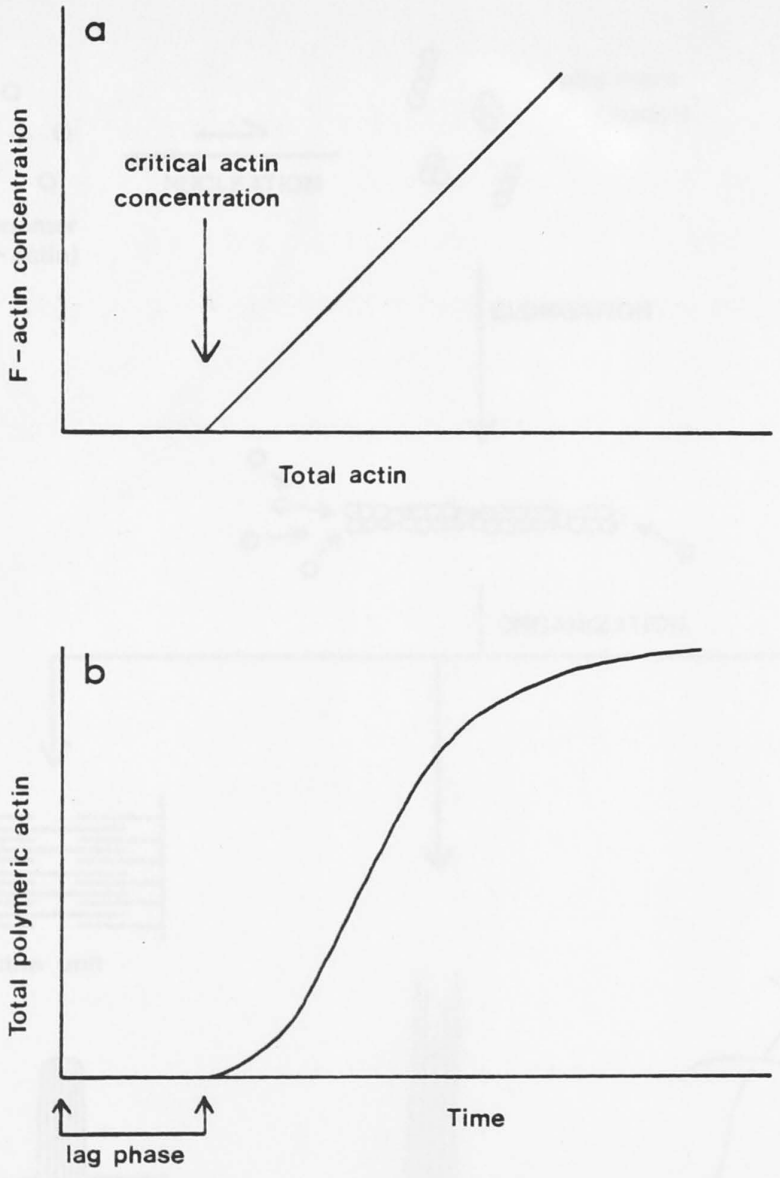


FIGURE 4: Kinetics of actin polymerization.

(a) The steady-state F-actin concentration as a function of the total actin concentration. (b) Time course of salt-induced actin self-assembly.

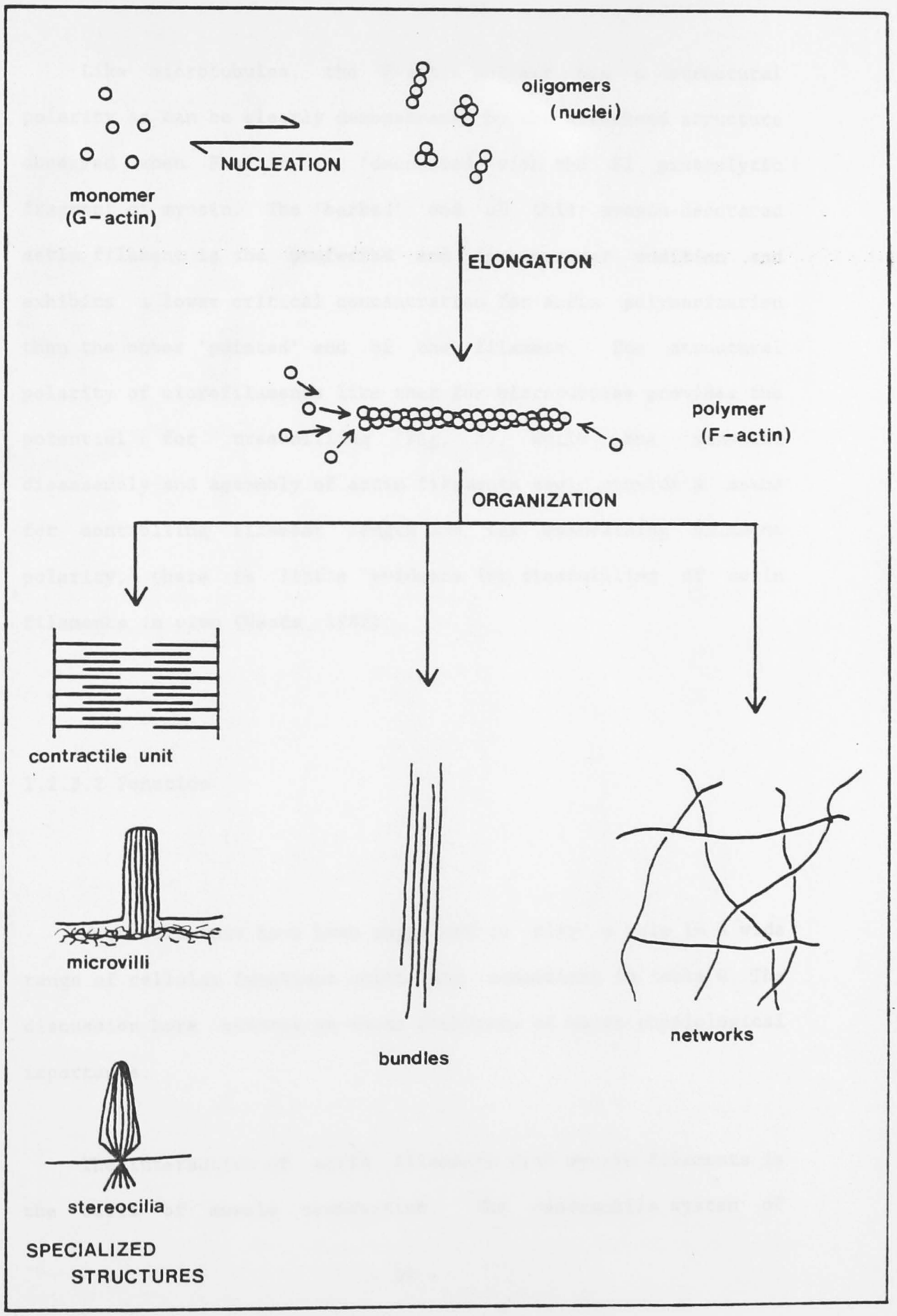


FIGURE 5: Levels of actin organization.

Like microtubules, the F-actin polymer has a structural polarity as can be clearly demonstrated by the arrowhead structure observed when F-actin is 'decorated' with the S1 proteolytic fragment of myosin. The 'barbed' end of this myosin-decorated actin filament is the preferred end for monomer addition and exhibits a lower critical concentration for actin polymerization than the other 'pointed' end of the filament. The structural polarity of microfilaments like that for microtubules provides the potential for treadmilling (Fig. 3). While the constant disassembly and assembly of actin filaments could provide a means for controlling filament length and for maintaining filament polarity, there is little evidence for treadmilling of actin filaments *in vivo* (Weeds, 1982).

1.2.3.2 Function

Microfilaments have been suggested to play a role in a wide range of cellular functions which are summarized in table 4. The discussion here centres on those functions of major physiological importance.

The interaction of actin filaments with myosin filaments is the basis of muscle contraction. The contractile system of

TABLE 4: Functions of the microfilament system.

Function	References
1. Contractile, motile and locomotive functions.	
* amoeboid motion	1, 2, 3, 4
* intracellular movement of secretory granules, organelles and chromosomes	5, 6, 7
* wound healing	8
* muscle contraction	9
* cytokinesis	1, 2, 3, 10
* tumour invasion	11
2. Supportive functions.	
* cytoplasmic consistency	5, 12
* support of cell shape	1, 13, 14, 15
* support of specific structures such as sterocilia and microvilli	16, 17
* linkage of cellular components	5
3. Modulation of cell surface activities.	
* endocytosis, pinocytosis, phagocytosis	10
* control of dendritic spine shape to modulate synaptic efficiency	18
* induction of surface specialization (synaptic clusters; coated pits)	3, 10, 17, 19
* capping	10, 19, 20
* modulation of response to hormone-receptor binding	19, 21
* control of transbilayer movement and fluidity of membrane lipids	19, 22, 23, 24
* control of mobility of integral membrane proteins	19, 25
* cell adhesion	17, 26, 27

References

1: Clarke & Spudich (1977); 2: Yumura *et al.* (1984); 3: Geiger (1983);
4: Glacy (1983); 5: Stossel (1984); 6: Brady *et al.* (1984);
7: Drenckhahn & Mannherz (1983); 8: Gordon *et al.* (1982);
9: Alberts *et al.* (1983); 10: Oliver *et al.* (1983);
11: Denk & Krepler (1982); 12: Sato *et al.* (1985); 13: Haest (1982);
14: Bennett (1985); 15: Gratzer (1983); 16: Mooseker *et al.* (1984);
17: Geiger (1985); 18: Cumming & Burgoyne (1983); 19: Nicolson (1976);
20: Heath (1983); 21: Landreth *et al.* (1985);
22: Comfurius *et al.* (1985); 23: Op den Kamp *et al.* (1985);
24: Franck *et al.* (1985); 25: Painter *et al.* (1985);
26: Hynes *et al.* (1981); 27: Chen & Singer (1982).

muscle, and its regulation by calcium in conjunction with the specialized regulatory proteins, tropomyosin and troponin, is well understood (Eisenberg & Greene, 1980; Alberts *et al.*, 1983). In non-muscle cells, a contractile system based on the interaction of myosin with actin filaments has been suggested to be important in the provision of the motile force for chromosomal separation and organelle movements (Weeds, 1982; Brady *et al.*, 1984). During cytokinesis, at the end of mitosis, a contractile ring formed from actin filaments encircles the dividing cell and, presumably, attaches to the plasma membrane so that contraction produces the cleavage furrow leading to separation of the two daughter cells (Geiger, 1983; Oliver *et al.*, 1983).

It is difficult to select one set of cytoskeletal elements as the primary regulator of cell shape (Oliver & Berlin, 1982) but it has been suggested that actin networks are a fundamental component for cytoplasmic structure in most cells (Brinkley, 1981). In many cells, including amoebae and echinoderm eggs, there are no intermediate filaments and few microtubules so that the jelly-like consistency of the cytoplasm can only be due to microfilaments (Pollard *et al.*, 1981; Stossel, 1984).

Microfilaments have a major role in the formation and maintenance of cellular structures. The luminal surfaces of the epithelial cells lining the intestine and the proximal tubules of the kidney are extensively convoluted forming large numbers of

microvilli thus providing a large surface area for absorbtion (Mooseker *et al.*, 1984). Each microvillus is supported by a core bundle of microfilaments anchored laterally, and at the microvillus tip, to the plasma membrane. At the base of the microvillus the core microfilament bundle extends into the terminal web, another microfilament-based structure which connects all the microvillus bundles (Mooseker *et al.*, 1984; Geiger, 1985). A very similar structure is found in the stereocilia of the cochlea and vestibule of the inner ear (Alberts *et al.*, 1983; Tilney & Tilney, 1984). In hepatocytes, a meshwork of microfilaments surrounds the bile canaliculi and may facilitate bile flow through cycles of contraction and relaxation (Denk & Krepler, 1982).

The mammalian erythrocyte endures considerable stress in passing through narrow capillaries. The strength and elasticity of the erythrocyte and its unique discoid shape are due to the presence of a two-dimensional lattice that underlies and is linked to the plasma membrane. The lattice consists of short actin filaments crosslinked by tetramers of an associated protein, spectrin. Spectrin binds to another protein, ankyrin, which in turn binds to an integral membrane protein, Band 3, the anion transporter, thus anchoring the entire lattice to the membrane (Haest, 1982; Gratzer, 1983; Bennett, 1985).

During platelet activation, a reorganization of the

microfilaments, as well as the marginal band of microtubules, is associated with the dramatic change in platelet shape (Pribluda & Rotman, 1982; Geiger, 1983; Feinstein *et al.*, 1985) which is essential for platelet aggregation and therefore clot formation. A similar shape change involving microfilaments is seen in the fertilization of eggs such as those of sea urchins (Tilney & Jaffe, 1980). Cell shape is also influenced by microfilaments through their involvement in cell adhesion to substrata and to neighbouring cells (Hynes *et al.*, 1981; Geiger, 1985). Intracellular actin filaments may be physically connected to extracellular fibronectin fibrils via a structure termed the fibronexus (Birchmeier *et al.*, 1981; Hynes *et al.*, 1981; Chen & Singer, 1982). Of the cytoskeletal features, only stress fibres (bundles of parallel actin filaments seen in most normal cells in culture) can be related to adhesion. Agents causing rounding and detachment of cells first disperse the stress fibres whereas microtubules and intermediate filaments change later along with cell shape (Badley *et al.*, 1980).

Several functions in which microfilaments may be involved are likely to be important to the development of malignancy. In particular, motile functions may be important to tumour invasion and metastasis. Disruption of the microfilament system may result in altered membrane activities which could, in turn, affect the interaction between the tumour cell and the external environment. This aspect of microfilament function in relation to malignancy

will be discussed further in section 1.3.2.

1.2.3.3 Microfilament-Associated Proteins

The diversity of cellular structures and functions postulated to involve microfilaments is matched by the plethora of actin-binding proteins capable of influencing actin organization at any of the steps involved in going from actin monomer to multi-filamentous structures such as the microvillus skeleton. These actin-binding proteins have been grouped into various functional categories (Schliwa, 1981; Weeds, 1982; Stossel, 1984; Pollard & Cooper, 1986), and will be discussed here in the categories listed in table 5.

In muscle cells microfilaments are involved in a highly organized stable structure that forms the contractile apparatus. The actins of muscle and non-muscle cells are structurally and chemically very similar; the difference in their cellular organization and, therefore, function is probably due to differences in the regulatory proteins of muscle and non-muscle cells. Because the regulatory proteins of muscle cells are so highly specialized to the contractile mechanism (Adelstein & Eisenberg, 1980), this discussion will concentrate on the actin

TABLE 5: Actin regulatory proteins.

Functional category	Examples
1. Actin monomer-sequestering	profilin
2. Actin filament nucleating, capping and severing	gelsolin, severin, fragmin, destrin
3. Actin filament stabilizing and crosslinking	tropomyosin, fimbrin, spectrin
4. Proteins which link actin filaments to other cellular structures	ankyrin, vinculin (?) 110,000 M_r protein

regulatory proteins of non-muscle cells.

Actin Monomer-Sequestering Proteins

In all non-muscle cells, the concentration of actin monomer is much higher than would be expected from the critical concentration for polymerization under *in vivo* ionic conditions (Carlsson *et al.*, 1977; Shimizu & Obinata, 1986). This is probably due in part to the action of monomer-sequestering proteins like the profilins, which form a stoichiometric complex with G-actin and inhibit its polymerization (Weeds, 1982; Nishida *et al.*, 1984a; Ozaki & Hatano, 1984; Pollard & Cooper, 1984). The formation of a profilin-actin complex (profilactin) may provide a mechanism for the storage of actin and controlled turnover of microfilaments (Carlsson *et al.*, 1977). At steady state G-actin is in equilibrium with F-actin and profilactin, thereby introducing a sensitive mechanism for regulating the amount of actin polymer and monomer within a cell (Tobacman *et al.*, 1983; Ozaki & Hatano, 1984).

Vertebrate profilin binds to phosphatidylinositol and several other anionic phospholipids and actin has a lower affinity for the profilin-lipid complex than free profilin. This interaction between lipid and profilin may therefore provide a mechanism for the regulation of profilin activity. For example, it is possible that the turnover of phosphoinositides that follows the binding of

TABLE 6: Actin monomer-sequestering proteins

Protein	M_r	Source	References
profilin	12,000-16,000	many mammalian tissues *Acanthamoeba* sea urchin eggs	1, 2, 3 4, 5, 6
DNase I	63,000	pancreatic secretions	7
Vitamin D-binding protein	58,000	plasma	8, 9, 10

References

1: Carlsson *et al.* (1977); 2: Ozaki & Hatano (1984);
3: Nishida *et al.* (1984a); 4: Tobacman *et al.* (1983);
5: Pollard & Cooper (1984); 6: Kaiser *et al.* (1983);
7: Blikstad *et al.* (1978); 8: Haddad *et al.* (1984);
9: Haddad *et al.* (1985); 10: Lees *et al.* (1984).

certain ligands to cell surface receptors (see Fig. 1) may release actin monomers bound to profilin, thereby resulting in the site specific polymerization of actin (Lassing & Lindberg, 1985; Europe-Finner & Newell, 1986; Pollard & Cooper, 1986).

The vitamin D-binding protein, present in plasma in high concentrations (Haddad *et al.*, 1985), and the enzyme deoxyribonuclease I (DNase I) both form high affinity stoichiometric complexes with G-actin preventing actin polymerization. However, the biological significance of these interactions is not clear (Pollard & Cooper, 1986).

Actin Filament Nucleating, Capping and Severing Proteins

Nucleation results from the stabilization and promotion of the formation of actin nuclei (Pollard & Cooper, 1986) thereby reducing or eliminating the lag phase in the polymerization time course (Fig. 4). Severing activity results in the shortening of actin filaments by a mechanism that seems to involve the cutting or breaking of the actin filaments. Capping proteins bind to one end of an actin filament preventing further incorporation of actin monomer at that end. Because of the polar nature of actin filaments, the critical concentration for actin polymerization is different at the two filament ends. Capping of one end of a filament will cause the apparent critical concentration to change to that of the opposite end. All but a few capping proteins bind

TABLE 7: Actin filament nucleating, capping and severing proteins.

Protein	M_r	Source	References
gelsolin	90,000	macrophages platelets many mammalian cell types	1, 2 3 - 7 8 - 13
brevin	90,000	plasma	12, 14 - 17
villin	95,000	brush border epithelia toad oocytes	18 - 20 21
severin	40,000	*Dictyostelium discoideum*	22, 23
fragmin	42,000	*Physarum plasmodium*	24, 25, 26
SU45	45,000	sea urchin eggs	27, 28, 29
cap 42(a+b)	2x42,000 (heterodimer)	*Physarum polycephalum*	25, 30, 31
-	41,000	macrophages	32
depactin	20,000	starfish oocyte	33
destrin	19,000	brain, kidney, starfish oocytes	34, 35
-	18,000	ascites hepatoma	36
-	17,000	echinoderms	34
actophorin	15,000	*Acanthamoeba*	37

References

1: Yin & Stossel (1979); 2: Yin & Stossel (1980);
3: Kurth *et al.* (1983); 4: Olomucki *et al.* (1984);
5: Lind *et al.* (1982); 6: Bryan & Coluccio (1985);
7: Markey *et al.* (1982); 8: Petrucci *et al.* (1983);
9: Kanno *et al.* (1985); 10: Nelson & Boyd (1985);
11: Yin *et al.* (1981b); 12: Yin *et al.* (1984);
13: Carron *et al.* (1986); 14: Wilkins *et al.* (1983);
15: Harris & Gooch (1981); 16: Harris & Weeds (1984);
17: Thorstensson *et al.* (1982); 18: Mooseker (1984);
19: Hesterberg & Weber (1983); 20: Glenney *et al.* (1982);
21: Corwin & Hartwig (1983); 22: Yamamoto *et al.* (1982);
23: Giffard *et al.* (1984); 24: Hasegawa *et al.* (1980);
25: Maruta *et al.* (1984); 26: Hatano *et al.* (1982);
27: Hosoya & Mabuchi (1984); 28: Wang & Spudich (1984);
29: Coluccio *et al.* (1986); 30: Maruta *et al.* (1983);
31: Maruta & Isenberg (1983); 32: Southwick & DiNubile (1986);
33: Mabuchi (1983); 34: Nishida *et al.* (1985);
36: Nishida *et al.* (1984b); 37: Ohta *et al.* (1984);
38: Cooper *et al.* (1986).

to the end of preferred assembly (the barbed end). At steady state this causes the critical concentration to increase and disassembly to be favoured until a new equilibrium is reached. The end result is numerous short actin filaments with capped barbed ends (Pollard & Cooper, 1986). Some actin-binding proteins, such as gelsolin, under certain conditions, may exhibit nucleating, capping and severing activities.

Transformations between "sol" (colloidal solution) and "gel" (a jellylike form of matter intermediate between solid and liquid) states have been suggested to be important for cytoplasmic structure and movement of peripheral cytoplasm in many eukaryotic cells (Stossel *et al.*, 1981; Pollard & Cooper, 1986). Solutions of filaments, formed from highly purified actin, possess both viscous and elastic properties, where viscosity is defined as the resistance to flow and elasticity as the storage of mechanical energy (Pollard & Cooper, 1986). The viscoelastic nature of F-actin solutions is due to the entanglement of the filaments (Stossel *et al.*, 1981) and, probably, also to weak interactions between filaments (Sato *et al.*, 1985; Pollard & Cooper, 1986). However, to form the high apparent viscosity (gel state) of peripheral cytoplasm, cross-linking of actin filaments into a continuous three-dimensional network is necessary (Yin & Stossel, 1982; Stossel, 1984).

Gelation induced by the cross-linking of actin filaments will

be discussed further in the section on actin cross-linking proteins. The reverse process, solation can, in principle, be achieved by several mechanisms: (1) removal of the cross-links (there is little evidence for this), (2) depolymerizing the actin polymers and (3) decreasing the actin filament length distribution without altering the relative amounts of actin monomers and polymers (Yin & Stossel, 1982).

Gelsolin, a 90,000 M_r protein that has been isolated from a wide variety of vertebrate cell types (Table 7) exhibits activities towards actin that could result in the solation of actin gels by the third mechanism above. Gelsolin, in the presence of micromolar concentrations of free calcium, severs actin filaments, nucleates actin polymerization and caps the barbed end of the filaments. The result of this combined severing, capping and nucleating activity is a reduction in the average length of actin filaments (Yin *et al.*, 1981a) thus causing solation of actin gels. Decreasing the calcium concentration rapidly reverses solation (Yin *et al.*, 1980). The sensitivity of gelsolin activity to calcium confers a regulatory mechanism on the solation of actin gels (Yin & Stossel, 1979). Gelsolin activity may be important in platelet activation (Kurth & Bryan, 1984) and the regulation of cell shape in response to physical agents such as glucocorticoids (Lanks & Kasambalides, 1983).

The mechanism of gelsolin action has been extensively studied

(Yin & Stossel, 1982; Bryan & Kurth, 1984; Kurth & Bryan, 1984; Bryan & Coluccio, 1985; Kwiatkowski *et al.*, 1985; Hwo & Bryan, 1986) and a number of proteins with similar calcium-sensitive activities have been identified (Bader *et al.*, 1986) including brevin (also called plasma gelsolin and actin depolymerizing factor) which is a secreted form of gelsolin of slightly higher M_r (Doi & Frieden, 1984; Coue & Korn, 1985; Bryan & Hwo, 1986), severin, and fragmin.

Villin, a 95,000 M_r protein which is found primarily in microvilli of brush border epithelia such as in the small intestine and the proximal tubule cells of the kidney, has a structure similar to gelsolin (Matsudaira *et al.*, 1985) and in the presence of calcium is a capping/severing/nucleating protein (Bonder & Mooseker, 1983; Walsh *et al.*, 1984a, Walsh *et al.*, 1984b) but in the absence of calcium it is additionally an F-actin bundling protein. In the microvillus, villin is located within the microfilament bundles suggesting that its major function *in situ* is as a bundling protein (Verner & Bretscher, 1985). Microvilli contain large amounts of calmodulin which may, through its calcium-binding properties (Klee *et al.*, 1980), act as a buffer to maintain low concentrations of free calcium. This buffering mechanism would allow for the transport of calcium from the intestine into the microvilli without the induction of the calcium-dependent severing activity of villin which would cause dissolution of the microvillar microfilament bundles (Glenney &

Glenney, 1985).

In some instances, solation may be induced by the depolymerization of the actin polymers. A number of low molecular weight proteins have been isolated which cause a rapid depolymerization, in a calcium-insensitive manner, by apparently severing actin filaments and/or binding to actin monomer. This group of depolymerizing proteins includes destrin from porcine brain and kidney, depactin from starfish oocytes, an 18,000 M_r protein from ascites hepatoma cells and actophorin from *Acanthamoeba*. The relationship between these different proteins is unclear since comparative structural data (peptide maps, amino acid sequences etc.) is still limited (Cooper *et al.*, 1986; Pollard & Cooper, 1986).

An interesting example of regulation of capping activity occurs in *Physarum polycephalum*. The protein, cap42(a+b) consists of two 42,000 M_r subunits, cap42(a) and cap42(b). Cap42(a+b) caps and nucleates but does not sever F-actin and requires calcium for activity only when cap42(b) is phosphorylated (Maruta & Isenberg, 1983; Maruta *et al.*, 1984).

In addition to a role in regulating cytoplasmic consistency through solation, capping proteins may inhibit the 'treadmilling' of actin filaments *in vivo*, or regulate the links between the barbed ends of actin filaments and the plasma membrane. Actin

filaments in a variety of cell types are known to be associated with the plasma membrane with the pointed ends directed away from the membrane *in situ*. This directionality of assembly has been suggested to result from the presence of barbed end capping proteins associated with the membrane (Tsukita *et al.*, 1985).

Actin Filament Stabilizing and Cross-linking Proteins

The intracellular distribution of the non-muscle tropomyosins is indicative of their role in the stabilization of F-actin. Tropomyosin is found associated with stress fibres of cultured cells (Sanger *et al.*, 1983) and with actin filaments in the terminal web underlying microvilli (Mooseker *et al.*, 1984) but tropomyosin is not present in highly motile areas such as membrane ruffles (Payne & Rudnick, 1984) where there is a dynamic microfilament organization. The presence of tropomyosin protects actin filaments against the severing action of gelsolin (Fattoum *et al.*, 1983; Payne & Rudnick, 1984) and the low molecular weight depolymerizing proteins such as destrin (Nishida *et al.*, 1985). In addition, tropomyosin reduces the flexibility of F-actin (Payne & Rudnick, 1984). The non-muscle tropomyosins are composed of two alpha-helical subunits of approximately 30,000 M_r and, although they exhibit amino acid sequence homology with muscle tropomyosin, they tend to be smaller molecules (Cote, 1983; Fattoum *et al.*, 1983; Giometti & Anderson, 1984). As many as five tropomyosin isoforms may be present in the cytoplasm of non-muscle cells

TABLE 8: Actin filament stabilizing and crosslinking proteins.

Protein	M_r	Source	References
tropomyosin	2x30,000 dimer	ubiquitous	1, 2, 3, 4
villin	95,000	microvilli of brush border epithelia	see table 7
fimbrin	68,000	vertebrate cells	5, 6
-	55,000	HeLa cells	7
actin-binding protein	270,000	variety of cell types	8, 9, 10
filamin	270,000	vertebrate cells	11, 12, 13
alpha-actinin	95,000	ubiquitous?	12, 14, 15, 16
actinogelin	115,000	variety of cell types	11, 17, 18
spectrin	tetramer of 240,000 and 220,000 subunits	erythrocytes	19, 20, 21
TW260/240	tetramer of 260,000 and 240,000 subunits	terminal web of microvilli	19, 22
caldesmon	140,000/80,000	smooth muscle platelets fibroblasts	23, 24, 25 26 27, 28

References

1: Cote (1983); 2: Giometti & Anderson (1984); 3: Fattoum *et al.* (1983);
4: Payne & Rudnick (1984); 5: Bretscher (1981);
6: Weber & Glenney (1981); 7: Yamashiro-Matsumura & Matsumura (1985);
8: Corwin & Hartwig (1983); 9: Sutoh *et al.* (1984);
10: Rosenberg *et al.* (1981); 11: Mimura & Asano (1979);
12: Langanger *et al.* (1984); 13: Weihing (1985);
14: Burridge & Feramisco (1981); 15: Jockusch & Isenberg (1981);
16: Mabuchi *et al.* (1985); 17: Mimura & Asano (1981);
18: Ohtaki *et al.* (1985); 19: Howe *et al.* (1985); 20: Brenner & Korn (1980);
21: Fowler *et al.* (1981); 22: Pearl *et al.* (1984);
23: Bretscher & Lynch (1985); 24: Maruyama *et al.* (1985);
25: Ngai & Walsh (1985); 26: Dingus *et al.* (1986);
27: Sobue *et al.* (1985); 28: Owada *et al.* (1984).

(Matsumura *et al.*, 1983; Giometti & Anderson, 1984; Lin *et al.*, 1985). The various isoforms may differ in the affinity with which they bind to actin filaments (Keiser & Wegner, 1985; Lin *et al.*, 1985; Matsumura & Yamashiro-Matsumura, 1986) suggesting that the various isoforms could have differing functions and intracellular distributions. This will be discussed further in relation to malignant transformation in section 1.3.2.

Tropomyosin is an example of a protein able to bind laterally to actin filaments, but without linking filaments together. Cross-linking proteins, by binding to the sides of more than one actin filament, cause the filaments to be organized into bundles or two- and three-dimensional networks. As has been discussed, the microvillus protein, villin, has bundling activity in the absence of calcium while in the presence of calcium it exhibits capping/severing/nucleating activity. However, the major bundling protein of the microvillus is thought to be fimbrin (Mooseker *et al.*, 1984). Fimbrin induces the formation of relatively straight bundles (Bretscher, 1981) suggesting it confers rigidity on the bundle. This is consistent with its *in vivo* localization in straight, highly organized microfilament bundles such as those present in microspikes, stereocilia and microvilli (Bretscher, 1981; Weber & Glenney, 1981).

A number of proteins have been isolated which are able to cross-link actin filaments into a three-dimensional network

thereby inducing gelation. An abrupt transition between sol and gel states of polymeric actin takes place at a critical density of cross-linker, defined as the ratio of the critical number of cross-links to the number of actin monomers in the polymer being cross-linked (Maruyama *et al.*, 1980; Yin & Stossel, 1982). The formation of an actin network by cross-linking proteins such as Actin-Binding Protein (ABP), filamin and actinogelin appears to obey this classic network theory (Yin & Stossel, 1982; Weihing, 1985). The cross-linking activity of ABP and filamin is calcium-insensitive (Mimura & Asano, 1979) and hence the degree of gelation induced depends only on the concentration of cross-linking protein and the number and length of the actin filaments being cross-linked. Gelation caused by actinogelin is inhibited by micromolar concentrations of Ca^{2+} (Mimura & Asano, 1981; Ohtaki *et al.*, 1985) and, at least in some cells, gelation by ABP can have calcium sensitivity conferred on it by the action of another protein, caldesmon.

Caldesmon has been isolated as a 140,000 M_r protein from smooth muscle and an 80,000 M_r protein from platelets, fibroblasts and other cultured cell lines (Owada *et al.*, 1984; Kakiuchi, 1985). The relationship between the different molecular weight forms of caldesmon is uncertain and there is conflicting evidence as to whether the protein is, in its native form, a dimer or a monomer (Dingus *et al.*, 1986). Caldesmon is localized along stress fibres and in leading edges of the cell (Bretscher & Lynch, 1985).

Although caldesmon itself has no effect on F-actin organization, the binding of caldesmon to F-actin inhibits gelation induced by actin-binding protein (Maruyama *et al.*, 1985; Sobue *et al.*, 1985). Caldesmon only binds to F-actin in the absence of calcium; in the presence of calcium, caldesmon binds to calmodulin and is dissociated from F-actin (Owada *et al.*, 1984; Kakiuchi, 1985; Maruyama *et al.*, 1985; Sobue *et al.*, 1985).

The erythrocyte membrane skeleton is a two-dimensional network of short actin filaments cross-linked by spectrin, a tetramer of 240,000 and 220,000 M_r subunits (Haest, 1982; Gratzer, 1983; Bennett, 1985). In the terminal web of brush border microvilli, the actin filaments are cross-linked by TW260/240, a protein very similar to spectrin but containing a different beta subunit (Howe *et al.*, 1985). A range of other spectrin-like proteins have been identified in non-erythroid tissues (Sutoh *et al.*, 1984; Bennett, 1985; McOsker & Bretscher, 1985). However, the injection of anti-spectrin antibodies into non-erythroid cells causes precipitation of spectrin and disruption of intermediate filaments but not microfilaments suggesting that in these cells spectrin is not directly involved in the organization of microfilaments (Mangeat & Burridge, 1984b). Spectrin is phosphorylated (Haest, 1982) but there is no direct evidence for any effect of this phosphorylation on spectrin function (Bennett, 1985). Spectrin is a calmodulin-binding protein making calcium-mediated regulation of its activity a possibility

(Kakiuchi, 1985).

The muscle form of alpha-actinin, a 100,000 M_r homodimer (Jockusch & Isenberg, 1981) binds to F-actin in a calcium-insensitive manner (Burridge & Feramisco, 1981) and is localized exclusively in the Z-lines of the sarcomere (Lazarides & Burridge, 1975). The non-muscle form is a similar, but distinct (Burridge & Feramisco, 1981) 95,000 M_r protein and its binding to F-actin is inhibited by micromolar concentrations of calcium (Burridge & Feramisco, 1981; Bennett *et al.*, 1984; Mangeat & Burridge, 1984a; Landon *et al.*, 1985). Alpha-actinin cross-links actin filaments into parallel bundles and although it has been suggested that it may also form gels (Jockusch & Isenberg, 1981; Landon *et al.*, 1985) this probably does not occur at 37^{o}C (Bennett *et al.*, 1984). Alpha-actinin is present in a wide variety of cells (Bennett *et al.*, 1984) bound along the length of actin filaments in stress fibres (Langanger *et al.*, 1984), in membrane ruffles (Weber & Osborn, 1982) and in the terminal web of microvilli (Mooseker *et al.*, 1984). Alpha-actinin is concentrated in adhesion plaques, the regions of the plasma membrane where microfilament bundles terminate and where the cell is attached most strongly to the substratum. This concentration of alpha-actinin in adhesion plaques suggests a role for alpha-actinin in the attachment of microfilament bundles to the membrane (Lazarides & Burridge, 1975; Jockusch & Isenberg, 1981).

Proteins Linking Actin Filaments to Other Structures

While it is becoming clear that the linkage of microfilaments to the plasma membrane is a general phenomenon, the erythrocyte remains the only cell for which the details of the linkage are understood (Pollard & Cooper, 1986). The linking protein is ankyrin which binds to both spectrin and band 3, the anion transporter, which is an integral membrane protein (Bennett, 1985). The band 4.1 protein, in addition to promoting the association between spectrin and actin (Spiegel *et al.*, 1984), also binds to the membrane through a high affinity site on the glycophorins, which are also integral membrane proteins, and a site of lower affinity on band 3 (Anderson & Marchesi, 1985). The association between band 4.1 and glycophorin may be regulated by a phosphoinositide cofactor (Anderson & Marchesi, 1985) while phosphorylation of ankyrin significantly reduces its affinity for spectrin tetramers (Lu *et al.*, 1985). Proteins with immunological cross-reactivity to both ankyrin and band 4.1 are present in non-erythroid cells (Cohen *et al.*, 1982; Davis & Bennett, 1984; Mangeat & Burridge, 1984a; Spiegel *et al.*, 1984; Bennett, 1985; Davies & Cohen, 1985) but their functions have not been determined.

Vinculin, a 130,000 M_r protein, and alpha-actinin are both major components of certain types of actin filament-membrane associations (Drenckhahn & Franz, 1986) including the

TABLE 9: Proteins linking actin filaments to other structures.

Protein	M_r	Source	References
ankyrin	215,000	erythrocyte brain	1, 2, 3 4
band 4.1	78,000 + 80,000	erythrocyte	1, 2
alpha-actinin	95,000	ubiquitous?	see table 8
110-K protein	110,000	microvilli	5, 6, 7
vinculin	130,000	wide variety of cell types	8, 9
meta-vinculin	150,000	chicken gizzard	10
talin	215,000	smooth muscle fibroblasts skeletal muscle	11 12, 13 14
connectin	70,000	fibrosarcoma cells	15
58kD/CAG	58,000 + 75,000	adenocarcinoma cells	16

References

1: Gratzer (1983); 2: Bennett (1985); 3: Haest (1982);
4: Davis & Bennett (1984); 5: Collins & Borysenko (1984);
6: Verner & Bretscher (1985); 7: Mooseker *et al.* (1984);
8: Jockusch & Isenberg (1981); 9: Rosenfeld *et al.* (1985);
10: Siliciano & Craig (1982); 11: Koteliansky *et al.* (1985);
12: Burridge & Connell (1983); 13: Mangeat & Burridge (1984a);
14: Sealock *et al.* (1986); 15: Brown *et al.* (1983);
16: Carraway *et al.* (1983).

cell-substratum focal contacts of cultured cells and the related adherens type of intercellular junctions (Burridge & Feramisco, 1980; Geiger *et al.*, 1981; Chen & Singer, 1982; Avnur *et al.*, 1983). Complexes of alpha-actinin, actin and lipid are formed *in vitro* in the presence of diacylglycerol and palmitic acid, and also *in situ* during the stimulation of blood platelet aggregation. This evidence supports the suggestion that alpha-actinin may be involved in the actin filament-membrane linkage (Burn *et al.*, 1985). However, vinculin is located closer to the membrane than is alpha-actinin and its association with the plasma membrane is actin-independent (Avnur *et al.*, 1983). It has been suggested that association of vinculin with the focal adhesion plaques induces the formation of actin bundles attached to these sites (Geiger *et al.*, 1980; Jockusch & Isenberg, 1981; Burridge & Mangeat, 1984). However, there are some discrepancies in the activities attributed to vinculin and, in particular, its ability to interact directly with actin (Burridge & Feramisco, 1981; Jockusch & Isenberg, 1981; Evans *et al.*, 1984; Koteliansky *et al.*, 1984). It now appears that the previously reported effects of vinculin on F-actin viscosity are due to a contaminant(s) in vinculin preparations (Mangeat & Burridge, 1984a; Rosenfeld *et al.*, 1985; Wilkins & Lin, 1986). Vinculin is generally considered to be a peripheral membrane protein (Geiger *et al.*, 1981) although it will interact with acidic phospholipids (Niggli *et al.*, 1986) and a larger protein, meta-vinculin (150,000 M_r) has been identified which is immunologically related to vinculin but has

the properties of an integral membrane protein (Siliciano & Craig, 1982).

Another protein, talin (215,000 M_r), is present in the focal adhesion plaques, cell margins and ruffling membranes of fibroblasts (Burridge & Connell, 1983) and the neuromuscular junction of skeletal muscle (Sealock *et al.*, 1986). Talin does not bind directly to actin but does interact with vinculin (Burridge & Mangeat, 1984). Alpha-actinin, vinculin and talin are all likely to have a role in the organization of actin filaments at the point of membrane interaction and may form part of a chain of integral and peripheral membrane proteins linking actin to the plasma membrane (Mangeat & Burridge, 1984a).

In microvilli the core bundle of actin filaments is connected laterally to the microvillus membrane by sidearms containing a complex of a 110,000 M_r protein and calmodulin (Coudrier *et al.*, 1981; Mooseker *et al.*, 1984; Verner & Bretscher, 1985). The 110,000 M_r protein-calmodulin complex binds actin in the absence of ATP and exhibits actin-activated ATPase activity (Collins & Borysenko, 1984; Pollard & Cooper, 1986). The complex has proved difficult to maintain in solution in aqueous buffers suggesting it may be an integral protein (Glenney & Glenney, 1984; Geiger, 1985) but there is also evidence suggesting it may be a peripheral protein (Verner & Bretscher, 1985).

A number of transmembrane proteins, including some cell surface receptors for growth factors and hormones, have been identified which bind to actin (Brown *et al.*, 1983; Rogalski & Singer, 1985; Carraway *et al.*, 1983). The cell surface receptor for laminin, which has also been called connectin (Brown *et al.*, 1983) and integrin (Tamkun *et al.*, 1986), binds to talin (Horwitz *et al.*, 1986) as well as to the extracellular matrix proteins fibronectin and laminin (Horwitz *et al.*, 1985). Integrin is one of a group of glycoproteins, each approximately 140,000 M_r and with solubility properties characteristic of integral membrane proteins, which probably constitute the transmembrane linkage between intracellular actin filaments and the extracellular matrix (Tamkun *et al.*, 1986). A 140,000 M_r protein isolated from microvilli and with properties of an integral membrane protein (Coudrier *et al.*, 1981; Mangeat & Burridge, 1984a) has been suggested to link the 110,000 M_r protein to the microvillar membrane and may also be a member of this group of 140,000 M_r glycoproteins.

Some investigators have suggested that actin itself may, under some circumstances, become inserted into the membrane but this is regarded as being unlikely (Geiger, 1985; Pollard & Cooper, 1986). It is possible that some of the proteins discussed in this section may also be important in attaching various membrane-bound organelles to the microfilaments but there is little evidence for this.

1.2.4 Intermediate Filaments

1.2.4.1 Structure

Intermediate filaments can be divided into at least five classes according to their biochemical make-up (Table 10). Despite their biochemical heterogeneity all intermediate filaments have a similar ultrastructure and the composite polypeptides are structurally related (Lazarides, 1982; Steinert *et al.*, 1984). Intermediate filament proteins are fibrous with a central alpha-helical 'rod' domain flanked by end domains of variable size and chemical character. The 'rod' domains may be primarily responsible for defining the basic filament structure while the end domains define the functions of the different filament types. Intermediate filaments are stable non-polar structures (Steinert & Steven, 1985), unlike microtubules and microfilaments, and little is known of the polymerization process. *In vitro* assembly from purified components has been achieved (Lazarides, 1982) but since all types of intermediate filaments are highly insoluble in isotonic buffers at physiological pH, it has been assumed that they are present in the cytoplasm only in the polymeric state (Pachter & Liem, 1985). While there are indications that

TABLE 10: Classes of intermediate filaments.

Class	Distribution	Composition
Cytokeratin	Epithelial cells	Heteropolymers of up to 10 polypeptides of 40,000 to 70,000 M_r
Vimentin	Mesenchymal cells; often expressed as second network in cultured cells	Homopolymers of vimentin (54,000 - 58,000 M_r)
Desmin	Muscle cells	Homopolymer of desmin (54,000 M_r)
Neurofilaments	Neurons	Composed of 68,000, 145,000 and 200,000 M_r polypeptides
Glial filaments	Astrocytes and oligodendrocytes but not neurons	Contain glial fibrillary acidic protein (51,000 M_r)

References

Lazarides (1982); David *et al.* (1983); Steinert *et al.* (1984).

intermediate filaments may at times undergo structural rearrangement (Jones *et al.*, 1985; Kitajima *et al.*, 1985) there is no evidence for a dynamic equilibrium between monomer and polymer forms such as occurs with microtubules and microfilaments (David *et al.*, 1983).

1.2.4.2 Function

The insolubility of intermediate filaments, plus the apparent lack of a dynamic equilibrium between monomeric and polymeric forms, suggests the intermediate filaments primarily play a supportive, structural role in the cell (Steinert *et al.*, 1984), a supposition that is supported by their intracellular distribution.

Intermediate filaments lie immediately below the microfilaments of the contractile ring during cytokinesis (Oliver *et al.*, 1983) and, in the intestinal epithelium, intermediate filaments form a skeleton underlying the terminal web. In the Purkinje fibres of the heart, intermediate filaments constitute 50% of all structural proteins, suggesting an important role in the preservation of tissue integrity during the severe tension of heart contraction (David *et al.*, 1983). The frequent association

of vimentin filaments with the cell nucleus suggests that they may support and constrain the nucleus to a specific location in the cell (Weber & Osborn, 1982; David *et al.*, 1983). In metaphase, the vimentin filaments form a cage around the mitotic spindle (Jones *et al.*, 1985; Kitajima *et al.*, 1985) thus excluding most organelles from the spindle area (David *et al.*, 1983).

All of the intermediate filament subunits are phosphoproteins but the effect of phosphorylation is unknown (Gard & Lazarides, 1982; Steinert *et al.*, 1984) and the incorporation of the proteins into filaments is independent of their phosphorylation state (Lazarides, 1982; Steinert *et al.*, 1982). Intermediate filaments may exist in a state of interdependence with microtubules: depolymerization of microtubules is accompanied by the collapse of some, but not all, types of intermediate filaments (Singer *et al.*, 1981) and there is some evidence of interaction between microtubules and the 200,000 M_r subunit of neurofilaments modulated by phosphorylation of the neurofilament subunit (Minami & Sakai, 1985).

The existence of cell lines, usually embryonic or 'dedifferentiated', devoid of intermediate filaments supports the notion that intermediate filaments are not involved in the basic cellular functions necessary for growth and proliferation but are related to special functions for the differentiated cell (Venetianer *et al.*, 1983).

1.2.4.3 Intermediate Filament-Associated Proteins

Just as the structure and functions of microtubules and microfilaments are regulated by associated proteins, it is becoming clear that intermediate filaments are likewise attended by associated proteins (Table 11). Some intermediate filament-associated proteins seem to be tissue specific or of limited distribution, suggesting a specialized function, whereas others are more widespread (Steinert *et al.*, 1984). Some, such as synemin, may crosslink intermediate filaments into networks (Yang *et al.*, 1985), while others, such as filaggrin (in cornified epidermal cells) may contribute to the rigidity of the intermediate filament network (Steinert *et al.*, 1984).

Another function of these proteins may be to link intermediate filaments to other cellular elements. Beta-internexin is identical to a protein found in preparations of MAPs from spinal cord (Napolitano *et al.*, 1985) suggesting it may be able to bind to both microtubules and intermediate filaments.

Desmocalmin is localized in desmosomes, specialized areas of cell-cell adhesion formed in a Ca^{2+}-dependent manner, with which

TABLE 11: Intermediate filament-associated proteins.

Protein	M_r	Source	References
synemin	230,000	chicken smooth muscle erythrocytes	1, 2 3
IFAP-300K	300,000	fibroblasts	4, 5
desmocalmin	240,000	epidermal cells	6
paranemin	280,000	skeletal muscle	1
alpha-internexin	66,000	nervous tissue	7
beta-internexin	70,000	ubiquitous?	8
NAPA-73	73,000	chick brain	9
p50	50,000	vimentin-associated	10
epinemin	45,000	vimentin-associated	11
filaggrin	30,000	keratin-associated	12

References

1: Lazarides (1982); 2: Granger & Lazarides (1980);
3: Granger & Lazarides (1982); 4: Lieska *et al.* (1985);
5: Yang *et al.* (1985); 6: Tsukita & Tsukita (1985);
7: Pachter & Liem (1985); 8: Napolitano *et al.* (1985);
9: Ciment *et al.* (1986); 10: Wang *et al.* (1983);
11: Lawson (1983); 12: Steinert *et al.* (1981).

are associated intermediate filaments. Desmocalmin binds to keratin filaments and in addition binds to calmodulin in a Ca^{2+}-dependent manner and may, therefore, regulate desmosome formation (Tsukita & Tsukita, 1985). The erythrocyte protein ankyrin will bind vimentin through the amino terminal domain thus linking vimentin to the erythrocyte plasma membrane (Georgatos & Marchesi, 1985; Georgatos *et al.*, 1985). Vimentin is not present in the mature mammalian erythrocyte but similar interactions with anykyrin-related proteins may be important in non-erythroid cells.

1.3 The Cytoskeleton in Disease

1.3.1 General

Given the diversity of functions in which the cytoskeleton is probably involved, it is surprising to find relatively few pathological conditions for which abnormalities of the cytoskeleton have been documented. This may reflect the central role played by the cytoskeleton in normal cellular functioning; major alterations are likely to be lethal.

In man, abnormalities in the energy-transducing interaction between dynein and microtubules results in the immotile cilia syndrome which is characterized by sperm immobility and ciliary dysfunction. The former causes sterility while the latter interferes with bronchial clearance and therefore leads to chronic lung infections (Denk & Krepler, 1982; David *et al.*, 1983; Rungger-Brandle & Gabbiani, 1983). In hepatocytes, diminution of microtubules in the course of ethanol intoxication may contribute to the retention of proteins normally secreted by these cells (Denk & Krepler, 1982).

Abnormalities in the actin cross-linking protein, spectrin, or the associated band 4.1 protein, cause the erythrocyte membrane skeleton to be weakened leading to fragile and/or abnormally shaped cells such as occur in hereditary spherocytosis and elliptocytosis. In both conditions the abnormal erythrocytes are accumulated in the spleen with resultant anaemia (Rungger-Brandle & Gabbiani, 1983; Bennett, 1985; Franck *et al.*, 1985).

Accumulations of intermediate filaments occur in a wide range of abnormal cells as a reflection of a diseased or injured state. Neurofilaments appear to proliferate in response to neurotoxins and neurological diseases such as giant axonal neuropathy, Wallerian and retrograde degeneration (Denk & Krepler, 1982; David *et al.*, 1983; Rungger-Brandle & Gabbiani, 1983). Accumulations of intermediate filaments are also seen in a hereditary disorder of mucous membranes and as Mallory bodies in the livers of alcoholics while certain forms of cardiomyopathy are considered secondary to disturbance in desmin metabolism (Denk & Krepler, 1982; David *et al.*, 1983).

1.3.2 The Involvement of the Cytoskeleton in Malignancy

The involvement of the cytoskeleton in cellular functions such as adhesion, motility, determination of cell shape, modulation of cell surface activities and, possibly, the initiation of cell division, all of which are altered in the malignant state, points to the potential importance of altered cytoskeletal function in the process of malignant transformation (Brinkley, 1982).

The clearest picture of alterations in microfilament organization associated with transformation is provided by the transformation of fibroblasts and other cells of mesenchymal origin (Brinkley, 1982). The stress fibres, parallel bundles of microfilaments, that are a dominant feature of normal fibroblasts in cell culture, are greatly reduced in size and number in transformed and malignant cell lines (Weber *et al.*, 1974; Carley *et al.*, 1981; Brinkley, 1982; Kahn *et al.*, 1983; Allred & Porter, 1979). That the loss of microfilament organization is related to the transformed phenotype is shown by studies of fibroblasts infected with transformation-defective, temperature-sensitive mutants of Rous sarcoma virus. Infected cells display an organized microfilament system with stress fibres at the

restrictive temperature but a shift to the permissive temperature induces disorganization of the microfilament system (Wang & Goldberg, 1976; Edelman & Yahara, 1976; Boschek *et al.*, 1981; Marchisio *et al.*, 1984). However, transformation-defective, temperature-sensitive viruses cannot be used to relate microfilament organization to tumorigenicity because of the requirement for non-physiological temperatures.

Much less is known about the organization of actin filaments in epithelial cells, both normal and transformed, but it does appear that an aberrant organization of actin filaments is associated with the transformation of epithelial cells (Bannikov *et al.*, 1982; Keski-Oja *et al.*, 1983) as well as of B lymphocytes (Caligaris-Cappio *et al.*, 1986). It has been suggested that the loss of actin organization is related to metastatic potential (Friedman *et al.*, 1985; Zachary *et al.*, 1986) and is not essential for tumorigenicity (Tsuchie *et al.*, 1986). The loss of actin-containing stress fibres has also been correlated with the acquisition of anchorage-independent cell growth (Pollack *et al.*, 1975; Kahn *et al.*, 1983) and a reduced adhesiveness to the substratum and other cells (Bannikov *et al.*, 1982). The relationship between adhesion, microfilament organization and cell shape is complex and it is not yet clear whether the reduced adhesiveness of transformed cells causes the alterations in microfilament organization or whether the altered microfilament organization leads to a weaker adhesiveness and altered cell shape

(Ben-Ze'ev, 1985).

Fibronectin, a 220,000 M_r glycoprotein present on the surface of many normal cells grown in culture and also in most basement membranes *in vivo*, is involved in cellular adhesion (Yamada, 1983; Gibson *et al.*, 1983). In most transformed cells fibronectin is either absent or greatly reduced in quantity (Yamada *et al.*, 1976; Marshall *et al.*, 1978; Der *et al.*, 1981; Singer, 1982). The fibronectin from transformed cells appears to be phosphorylated to a greater extent than that from normal cells, although the sites of phosphorylation are the same (Ali & Hunter, 1981) and there may be differences in the cell binding region (Borsi *et al.*, 1985). The significance of these changes is unknown.

The points of strongest contact between the cell and the substratum occur at the focal adhesion plaques (Singer, 1982). Fibronectin is excluded from the extracellular surface of these regions (Birchmeier *et al.*, 1981), but is concentrated in the region of close contact surrounding the focal adhesion plaque. The cytoplasmic surfaces of the focal adhesion plaques are the sites of membrane attachment for the bundles of microfilaments and since there is a strong correlation between the extracellular distribution of fibronectin fibrils and the intracellular distribution of actin filaments, a transmembrane association between the two systems seems likely (Hynes *et al.*, 1981; Singer & Paradiso, 1981; Singer, 1982; Gibson *et al.*, 1983). The attachment

of microfilament bundles at the focal adhesion plaques may be mediated by the proteins vinculin, talin and alpha-actinin. Transmembrane proteins such as integrin (Tamkun *et al.*, 1986) by interacting with extracellular fibronectin and intracellular talin (Horwitz *et al.*, 1986; Tamkun *et al.*, 1986) may complete the transmembrane linkage between the two systems.

Fibroblasts transformed with RSV exhibit an altered distribution of vinculin and alpha-actinin (David-Pfeuty & Singer, 1980) and the transforming protein of RSV, $pp60^{src}$, is localized in the focal adhesion plaques (Rohrschneider *et al.*, 1981; Nigg *et al.*, 1982). This suggests that the proteins of the focal adhesion plaque are likely targets for phosphorylation by $pp60^{src}$, a tryosine-specific protein kinase, with resultant disruption of the attachment of microfilaments to the membrane. Levels of phosphotyrosine in vinculin are significantly increased in cells transformed by RSV (Rohrschneider *et al.*, 1981; Sefton *et al.*, 1981; Ito *et al.*, 1983) but not in cells transformed by chemical carcinogens or SV40 virus (Sefton *et al.*, 1981). Further, the addition of fibronectin to RSV transformed chick embryo fibroblasts restores the cells to a near normal morphology and the fibronectin is incorporated into an extracellular matrix without any alteration in phosphotyrosine content of vinculin (Kellie *et al.*, 1986). Vinculin may also be phosphorylated on serine and threonine residues by protein kinase C, the proposed receptor for the phorbol ester tumour promoters (Werth *et al.*, 1983; Werth &

Pastan, 1984). The treatment of African green monkey kidney (BSC-1) cells with the phorbol ester 12-O-tetradecanoyl phorbol-13-acetate (TPA) induces the depletion of alpha-actinin followed by vinculin from the focal adhesion plaques, with concomitant disruption of the actin stress fibres (Meigs & Wang, 1986). Alpha-actinin contains negligible amounts of phosphate (Hunter, 1980) making it an unlikely substrate for phosphorylation while talin is phosphorylated by protein kinase C, but not, apparently, by $pp60^{src}$ or cAMP-dependent protein kinase (Litchfield & Ball, 1986).

The picture emerging from these data, therefore, is one of a transmembrane interaction between microfilaments and the extracellular matrix mediated by a chain of proteins, including alpha-actinin, vinculin, talin and integrin. Phosphorylation of vinculin or talin by $pp60^{src}$ or protein kinase C may disrupt this interacting system. However, elucidation of the functions of vinculin and talin will be needed before understanding of this system can be achieved.

Reduced levels of tropomyosin (Hendricks & Weintraub, 1981; Leonardi *et al.*, 1982) and changes in the expression of tropomyosin isoforms have been reported to occur in many types of transformed cells (Matsumura *et al.*, 1983; Lin *et al.*, 1985). The general pattern in these changes is that at least one of the isoforms of higher molecular weight is decreased or missing, and

the level of at least one of the isoforms with a lower molecular weight tends to be increased. Such an alteration of the relative amounts of tropomyosin isoforms could be significant given that the tropomyosin isoforms may differ in activity and intracellular distribution. Tropomyosin isoforms have been shown to differ in the affinity with which they bind to actin and the low, but not the high, molecular weight isoforms of tropomyosin are caused to dissociate from F-actin by the addition of a 55,000 M_r bundling protein from HeLa cells (Matsumura & Yamashiro-Matsumura, 1986). The presence of tropomyosin protects against the severing action of proteins such as gelsolin and destrin and, consequently, reduced levels of tropomyosin could lead to increased susceptibility of F-actin structures to actin filament severing proteins.

The expression of actin isoforms is variable with non-muscle cells containing beta- and gamma-actins in ratios ranging from 6:1 to 1:8 (beta:gamma) in different tissues and cell types (Otey *et al.*, 1986) while the major isoform of skeletal muscle is alpha-actin. The biological significance of this tissue-specific expression of actin isoforms remains unknown but there is some evidence indicating that certain physiological changes in cells can be correlated with alterations in a specific actin isoform. Stimulation of *Dictyostelium* amoebae with chemoattractants elicits a rapid accumulation of one of the three major actin isoforms of *Dictyostelium* in the Triton-insoluble cytoskeleton. Although the

significance of accumulation of the actin isoform in the Triton-insoluble cytoskeleton is unclear, this result does demonstrate one situation where a distinction between isoforms is made.

There have been several reports of altered ratios of actin isoforms in transformed cells (Leavitt *et al.*, 1980; Bravo *et al.*, 1981; Witt *et al.*, 1983; Goldstein & Leavitt, 1985; Leavitt *et al.*, 1985). In a series of chemically transformed human fibroblast cell lines increased malignant potential has been found to be accompanied by expression of mutant beta-actin genes. The mutant actin species differed from normal beta-actin by increased net negative charge, a reduced half-life in the cell, reduced ability to incorporate into the Triton-insoluble cytoskeleton, reduced affinity for DNase I and a faster rate of synthesis (Hamada *et al.*, 1981; Leavitt *et al.*, 1982; Kakunaga *et al.*, 1984). Expression of this variant actin is vastly reduced in cell lines of high metastatic potential (Taniguchi *et al.*, 1986). A transformed mouse cell line, sarcoma 180, also synthesizes large amounts of a variant actin that is more acidic than beta-actin and, while in these cells no apparent differences in localization of the variant actin were detected (Bravo *et al.*, 1981) and such a mutant actin has not been detected in any other transformed or malignant cells, these findings provide support to the hypothesis that alterations in microfilament function or organization may contribute to the process of malignant transformation. Given that

malignant cells, in general, exhibit a loss of differentiation, and that ratios of actin isoforms change with differentiation state, it is possible that the changes of actin isoform expression in transformed cells reflect their state of differentiation and not tumorigenicity *per se*.

The oncogene of the Gardner-Rasheed feline sarcoma virus, v-*fgr*, is homologous in part to a cytoskeletal actin gene and a tyrosine-specific protein kinase gene (Naharro *et al.*, 1984). Although the mechanism of action is yet to be elucidated, it is possible that the component with homology to actin may result in the targeting of actin-binding proteins for phosphorylation by the tyrosine protein kinase component (Naharro *et al.*, 1984).

The state of microtubule organization in transformed cells has been the subject of some controversy (Brinkley, 1982; Ben-Ze'ev, 1985). While initial studies demonstrated a decreased number of microtubules in transformed cells (Hynes, 1979), more recent studies have shown that transformed cells contain a highly organized network of microtubules (De Mey *et al.*, 1978; Der *et al.*, 1981; Sefton *et al.*, 1981). These discrepancies are due, at least in part, to the rounded morphology of transformed cells making assessment of microtubule organization difficult (Osborn & Weber, 1977). The treatment of epithelial African green monkey kidney (BSC-1) cells with the tumour promoter TPA, induces a rapid and reversible redistribution of actin and vinculin but the

integrity of microtubules and vimentin filaments is not affected by this treatment although their distribution is adjusted to the distorted cell shape. It therefore seems that cell shape influences microtubule organization and not *vice versa* (De Mey *et al.*, 1978). Since the microtubules are vitally important in the formation of the mitotic spindle, severe disruption of microtubule structure in transformed cells would be unexpected (Osborn & Weber, 1977) but subtle changes in function are possibly involved.

Changes in the intermediate filament network similarly do not appear to be critical to transformation. This is, perhaps, not surprising given that intermediate filaments are postulated to have a mostly structural function. In general, ultrastructural and immunological features of intermediate filaments are maintained during neoplastic transformation although accumulations of intermediate filaments have been noted in different types of tumours (Bannasch *et al.*, 1982; Keski-Oja *et al.*, 1983). The maintenance of cell-type specific expression of intermediate filaments has in fact proved to be a valuable tool in the histology and classification of tumours (David *et al.*, 1983). Metastases of solid tumours appear to retain the intermediate filaments characteristic of the primary tumour (Ben-Ze'ev, 1985) while the expression of vimentin filaments as a second intermediate filament network by some tumour cells may be interpretable in terms of a reversion to the embryonic behaviour

pattern (Lane *et al.*, 1983). The expression of intermediate filaments in transformed cells thus appears to reflect the differentiation state, rather than the malignant potential, of the cells.

1.4 Conclusions

The cytoskeleton is capable of interacting with the plasma membrane and consequently is potentially important for the transmission of information from the plasma membrane into the cytoplasm. The majority of oncogenes, as well as the phorbol ester tumour promoters, appear to disrupt the pathways by which control signals are usually transmitted into the cell. The disruption of cytoskeletal function may be another mechanism by which the pathway of signal transmission is altered in malignant cells. While there is little evidence to suggest that disruption of the microtubule and intermediate filament systems occurs in malignant transformation, there is considerable evidence associating alterations of microfilament organization with the development of malignancy.

The microfilament system is complex and disruption of organization and function could therefore occur by many mechanisms. Alterations in actin, the major structural protein, the balance of actin isoforms in the cell, the ratio of polymeric:monomeric actin or the total amount of actin in the cell could all conceivably result in an altered microfilament system.

However, the microfilament system includes a vast array of regulatory proteins, some of which are potentially regulated in their activity by phosphorylation state, levels of free calcium and/or competition with other proteins for binding sites on actin. Alterations in the amount or activity of any of these actin regulatory proteins could disrupt the interaction of microfilaments with other cellular components as well as the organization and functioning of the microfilament system. It must also be considered that alterations of the microfilament system may not be of primary importance to the development of the malignant phenotype but may be secondary to changes in adhesiveness, cellular morphology or differentiation state (Willingham *et al.*, 1977; Allred & Porter, 1979; Brinkley *et al.*, 1980).

Much of the evidence supporting the association of altered microfilament organization with the malignant state comes from systems in which transformation and tumorigenicity cannot be separated. Where the distinction has been made between the transformed and tumorigenic states it has been concluded that microfilament disorganization is not essential for expression of the transformed state (Willingham *et al.*, 1977). It is of interest, therefore, to assess microfilament organization in a system that allows distinction of transformed and tumorigenic states and in which changes in adhesion and cell shape are not likely to be a major factor influencing microfilament

organization. The HeLa/fibroblast somatic cell hybrid system satisfies these criteria.

The hypothesis formulated as the basis of this study is "that the normal organization of the microfilament system is disrupted during the transition from a transformed to a tumorigenic (malignant) state". The aim of this study is to test this hypothesis through examination of the microfilament system of HeLa/fibroblast hybrid cells and to investigate the mechanisms by which disruption of the microfilament system might occur.

Chapter 2

MICROFILAMENT ORGANIZATION AND TOTAL ACTIN CONTENT OF TUMORIGENIC AND NONTUMORIGENIC HUMAN SOMATIC CELL HYBRIDS.

2.1 Introduction

The cytoskeleton, through connections with integral components of the plasma membrane may play an important role in the process by which information is transmitted from the plasma membrane into the cytoplasm, or in the modulation of the cellular response to such signals. A common feature of malignant transformation is the interference with this transmission process and, consequently, alterations of cytoskeletal structure and function could be important in the expression of the malignant phenotype. There is evidence that alterations in the organization of the microfilaments, one of the three major filamentous components of the cytoskeleton, are associated with the acquisition of a transformed phenotype (see Chapter 1).

Much of the evidence indicative of a correlation between the loss of microfilament organization and malignant transformation comes from studies utilizing experimental systems, such as fibroblasts infected with transformation-defective, temperature-sensitive mutants of Rous sarcoma virus (RSV), in which it is not possible to distinguish between the transformed and tumorigenic states. Discrimination between these states is

advantageous since cellular characteristics, such as a loss of microfilament organization, associated with the transition from a transformed to a tumorigenic state may be more closely linked to the malignant phenotype than are characteristics acquired in the transition from a normal to a transformed state.

The HeLa/fibroblast hybrid cell system with its pairs of closely related tumorigenic and nontumorigenic cells is useful for a detailed examination of the microfilament system in relation to malignant transformation for a number of reasons. (1) The transformed and tumorigenic states can be distinguished, (2) the tumorigenic and nontumorigenic cells grow with approximately the same generation interval and therefore any biochemical differences can be expected to be related to the expression of tumorigenicity rather than the rate of growth and (3) the cells grow as adherent colonies with similar morphologies which not only simplifies microscopic comparison but also makes it unlikely that any differences in microfilament organization are related to cellular morphology and adhesiveness. The use of the HeLa/fibroblast hybrid cell system also minimizes some of the difficulties associated with studying cells from naturally occurring tumours. In particular, it is difficult to ascertain the cell type from which a tumour cell is derived and consequently which normal cell type the tumour cell should be compared with.

In earlier studies of the HeLa/fibroblast somatic cell hybrid

system, Stanbridge *et al.* (1982) noted a correlation between the disorganization of the microfilaments and the reappearance of tumorigenicity. The first stage of this study aims to document in more detail the organization of the microfilament system in the HeLa/fibroblast somatic cell hybrids using both microscopic and biochemical techniques.

2.1.1 Analysis of Microfilament Organization by Fluorescence Microscopy.

The most abundant phallotoxin of the *Amanita phalloides* mushroom, phallacidin, is a small bicyclic peptide consisting of seven amino acids. It contains as unusual thioether bridge between a cysteine and a tryptophan residue thus forming an inner ring structure (Fig. 6). Phallacidin binds strongly to F-actin (K_d=2x10^{-8}M) and stabilizes that structure but is unable to bind to monomeric G-actin (Barak & Yocum, 1981). NBD (4-chloro-7-nitrobenz-2-oxa-1,3-diazole) is a small fluorescent molecule which produces a brilliant yellow fluorescence when coupled to primary amines and excited by visible light. NBD has been coupled to phallacidin such that the fluorescent conjugate retains its high affinity for F-actin (Barak & Yocum, 1981). The small size, solubility in aqueous buffers and specificity of the

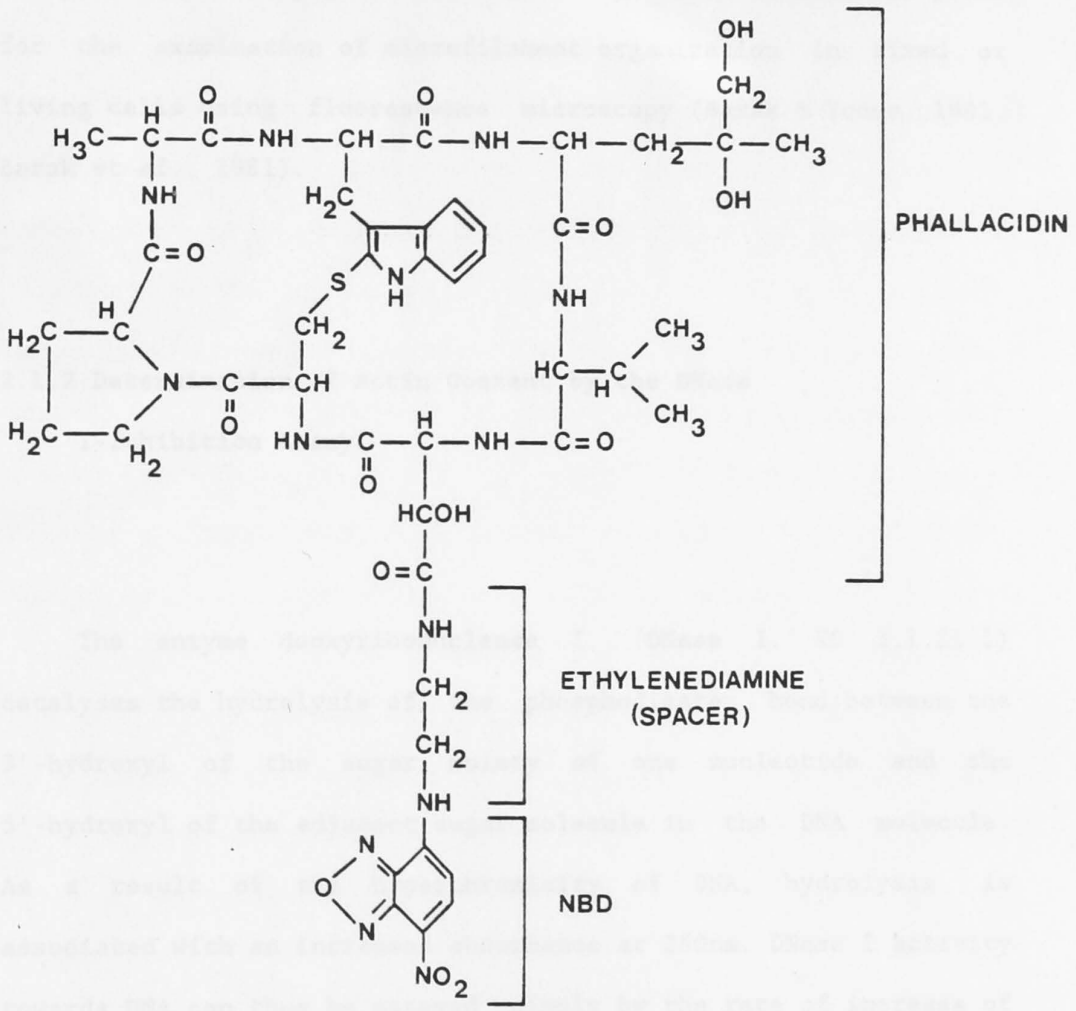


FIGURE 6: The structure of the fluorescent probe, NBD-phallacidin.

The fluorophore, NBD (4-chloro-7-nitrobenz-2-oxa-1,3-diazole) is coupled to the carboxylic acid residue of the phallacidin molecule through an ethylenediamine spacer.

(Adapted from Barak *et al.*, 1980)

NBD-phallacidin conjugate make it a very useful fluorescent probe for the examination of microfilament organization in fixed or living cells using fluorescence microscopy (Barak & Yocum, 1981; Barak *et al.*, 1981).

2.1.2 Determination of Actin Content by the DNase I-Inhibition Assay.

The enzyme deoxyribonuclease I (DNase I, EC 3.1.21.1) catalyses the hydrolysis of the phosphodiester bond between the 3'-hydroxyl of the sugar moiety of one nucleotide and the 5'-hydroxyl of the adjacent sugar molecule in the DNA molecule. As a result of the hyperchromicity of DNA, hydrolysis is associated with an increased absorbance at 260nm. DNase I activity towards DNA can thus be assayed simply by the rate of increase of absorbance at 260nm (Bergmeyer, 1974). Native monomeric actin binds stoichiometrically and with high affinity to DNase I and in so doing, inhibits the latter's activity towards DNA (Lazarides & Lindberg, 1974). The physiological significance of this high affinity interaction between monomeric actin and DNase I, if any, is unclear but it has proved invaluable in the development of several experimental techniques, notably affinity chromatography for the purification of actin and actin-binding proteins and the

assay of actin content by inhibition of DNase I activity.

The relationship between the degree of inhibition of DNase I activity and the concentration of actin monomer is linear for inhibition levels between 20% and 70% (Fig. 7). It is this relationship that is the basis of the DNase I-inhibition assay for measurement of the actin monomer content of cell extracts. While DNase I binds actin monomer, and the profilin-actin complex, with a high affinity (K_d=5x10^{8}M^{-1}), the affinity with which it binds to F-actin is much lower (K_d=1.2x10^{4}M^{-1}; Mannherz *et al.*, 1980). Consequently, through comparison with an appropriate standard curve (Fig. 7) the actin monomer content of lysed cell suspensions can be selectively determined (Blikstad *et al.*, 1978). Treatment of a sample of the lysed cell suspension with 0.75M-guanidine hydrochloride depolymerizes actin polymer to native actin monomer allowing the determination of the total actin content of the lysate.

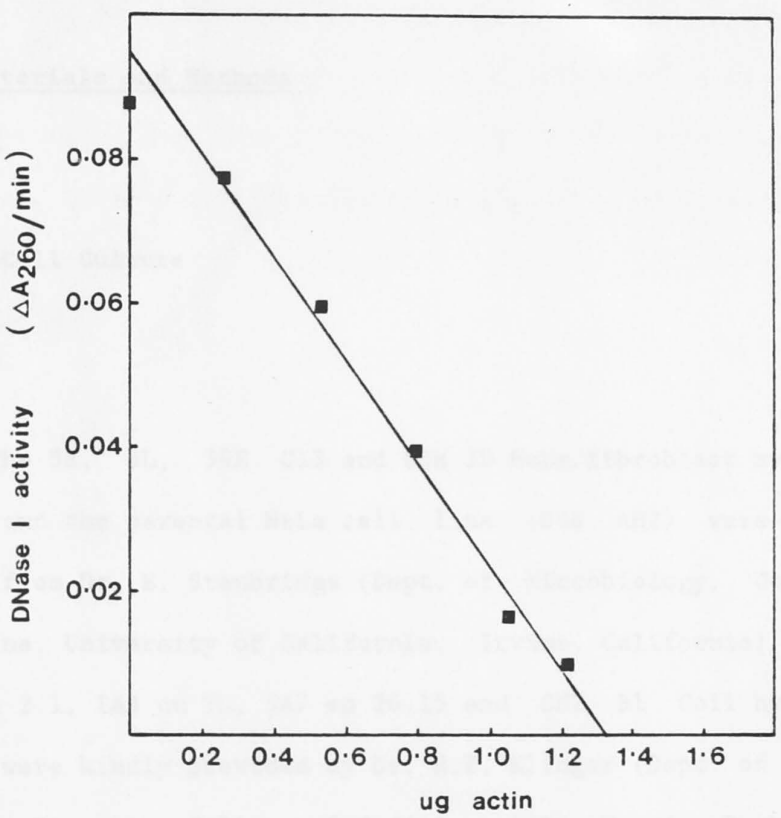


FIGURE 7: Inhibition of DNase I activity by monomeric actin.

A typical standard curve which can be used to calculate actin monomer content of a sample.

2.2 Materials and Methods

2.2.1 Cell Culture

The 5E, 5L, 39E C13 and ESH 39 HeLa/fibroblast hybrid cell lines and the parental HeLa cell line (D98 AH2) were generous gifts from Dr. E. Stanbridge (Dept. of Microbiology, College of Medicine, University of California, Irvine, California) while the IA3 cn 2.1, IA3 cn TG, 5A7 mp 26.15 and CN2 B1 Coll hybrid cell lines were kindly provided by Dr. H.P. Klinger (Dept. of Genetics, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York). The human fibroblast cell line (MRC-5) was obtained from Flow Laboratories and the bronchocarcinoma cell line (H.Ep.2) was obtained from the Sir William Dunn School of Pathology, Oxford, England. All cell lines were routinely tested for tumorigenicity by the injection of $5x10^6$ cells subcutaneously into nude mice. Cell lines that produced tumours within 2-3 weeks of inoculation were classified as tumorigenic. Cells were also regularly tested for contamination by mycoplasma using the method of Chen (1977).

All cell lines were cultured in DMEM (Dulbecco's Modified

Eagle's Medium) from Gibco. This was supplemented with 5%(v/v) foetal calf serum, 5%(v/v) newborn calf serum (except in the case of the MRC-5 cell line where 10%(v/v) foetal calf serum was used), 2mM pyruvate and antibiotics (50 units/mL penicillin G, 50 units/mL streptomycin sulphate and 180 units/mL neomycin sulphate). Incubation was at 37°C in a 5% CO_2 (in air) humidified atmosphere.

2.2.2 Microscopy

The microfilaments were fluorescently labelled with NBD-phallacidin (Molecular Probes Inc., Oregon, U.S.A.) essentially according to the method of Barak *et al.* (1980). Cells, grown on 12mm coverslips, were washed free of medium with phosphate-buffered saline (PBS: 7mM Na_2HPO_4, 3mM NaH_2PO_4, 137mM NaCl) containing 0.02% NaN_3 (PBS/NaN_3). The cells were fixed by immersion for 10min in freshly prepared 4% (w/v) paraformaldehyde in PBS/NaN_3, extracted with acetone at -20°C for 2-5min and air-dried. Each coverslip was covered with 30uL of PBS/NaN_3 containing 5ng NBD-phallacidin and incubated for 20min at room temperature. Controls were prepared by omitting the NBD-phallacidin from the procedure. After rinsing twice in PBS/NaN_3 and once in distilled water, the coverslips were mounted,

cell-side down, in PBS/NaN_3 plus 20% (v/v) glycerol and sealed with clear nail varnish. Slides prepared in this way can be stored in the dark at 4°C for at least 5 months without any noticeable loss of fluorescence.

Slides were examined under oil immersion using a Leitz Orthoplan Universal Largefield microscope with a Ploemopak 2 Fluorescence vertical illuminator (filter block I2; excitation range 450-490nm, emission above 515nm). For photography a Leitz Vario-Othomat camera system was used with Kodacolor VR1000 film.

2.2.3 Preparation of Rabbit Skeletal Muscle Actin

Rabbit skeletal muscle actin was prepared according to the method of Pardee & Spudich (1983) with the Sephadex G-150 modification of McLean-Fletcher & Pollard (1980). With this method, actin oligomers are eluted from the Sephadex column as a small peak preceding the main peak (Fig. 8). By pooling the middle and trailing fractions of the main actin peak, a preparation is obtained that is greater than 98% pure, as judged by polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate (SDS).

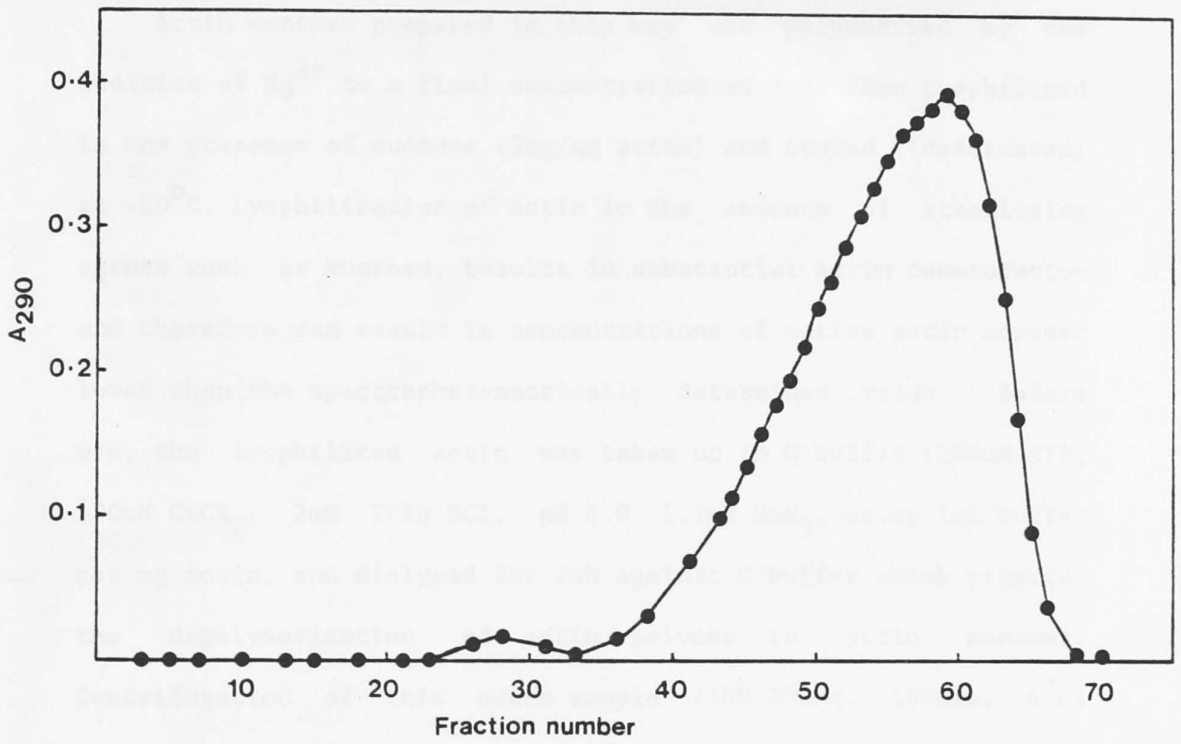


FIGURE 8: Preparation of rabbit skeletal muscle actin.

The major peak is monomeric actin, eluted from the Sephadex G-150 column in G-buffer (200uM ATP, 200uM $CaCl_2$, 2mM Tris.HCl, pH 8.0, 1.5mM NaN_3). Fractions 50 to 65 were combined to give a preparation of monomeric actin with a high degree of purity. Fractions 43 to 49 and fraction 66 were also combined to give a preparation of actin which, while pure, may contain some actin oligomers.

Actin monomer prepared in this way was polymerized by the addition of Mg^{2+} to a final concentration of 2mM, then lyophilized in the presence of sucrose (3mg/mg actin) and stored (dessicated) at -20°C. Lyophilization of actin in the absence of stabilizing agents such as sucrose, results in substantial actin denaturation and therefore can result in concentrations of native actin monomer lower than the spectrophotometrically determined value. Before use, the lyophilized actin was taken up in G-buffer (200uM ATP, 200uM $CaCl_2$, 2mM Tris.HCl, pH 8.0, 1.5mM NaN_3) using 1mL buffer per mg actin, and dialysed for 24h against G-buffer which promotes the depolymerization of actin polymer to actin monomer. Centrifugation of this actin sample (100,000xg, 180min, 4°C) removes denatured and polymeric actin, leaving a highly pure preparation of native monomeric actin in the supernatant. The concentration of actin in solutions prepared in this way was measured spectrophotometrically using an extinction coefficient of 0.63 absorbance unit at 290nm for a 1mg/mL solution (Houk & Ue, 1974).

2.2.4 Measurement of the Actin Content by the DNase I-Inhibition Assay.

The adherent cells were detached from the culture flasks by

incubation in PBS containing 0.025% (w/v) trypsin and 0.02% (w/v) EDTA (ethylenediaminetetra-acetic acid) for 10min at 37°C. Cells were pelleted by centrifugation (550xg, 4min), resuspended in growth medium and an aliquot removed for a cell count. Cell counts were performed in duplicate using the Trypan Blue exclusion method and a haemocytometer. The cells were pelleted, washed twice in PBS and the final cell pellet was resuspended in SF (Blikstad & Carlsson, 1982) lysis buffer (100mM NaF, 50mM KCl, 2mM $MgCl_2$, 1mM EGTA (ethyleneglycol bis-(aminoethyl)-tetra-acetic acid), 10mM K_2HPO_4, pH 7.0, 0.2mM dithioerythritol, 1M sucrose, 0.5% (w/v) Triton X-100). The total and monomeric actin contents of the lysed cell suspensions were then measured using the DNase I-inhibition assay using the procedure of Blikstad *et al.* (1978) with the modifications of Blikstad and Carlsson (1982). Standard curves for the inhibition of DNase I activity *versus* actin monomer concentration were always performed using monomeric rabbit skeletal muscle actin freshly prepared as described in section 2.2.3.

In order that the results obtained using the DNase I-inhibition assay reflect the *in vivo* situation as closely as possible, it is important that the cell lysis step causes minimal disruption to microfilament organization. Standard cell lysis procedures such as sonication and homogenization are unacceptable as both cause fragmentation and, subsequently, depolymerization of actin filaments. Choice of the buffer system must also be made

with care as some buffers, such as 10mM Tris.HCl, pH 8.0, 0.25M sucrose, 0.5% (w/v) Triton X-100, cause monomerization of greater than 90% of cellular F-actin during lysis, whilst other buffers favour polymerization after lysis. The lysis buffer used in this study was the buffer system (SF buffer) found by Blikstad & Carlsson (1982) to best stabilize actin monomer:polymer ratios. The actin monomer content and the total actin content of a cell suspension treated with SF lysis buffer was stable for about 30min and the results shown are means of three determinations made during this 30min period.

The total actin content of the lysed cell suspension was determined after treatment of a sample of the lysate with 0.75M-guanidine hydrochloride, which depolymerizes actin polymer to actin monomer without denaturation. Cell lysate samples incubated with guanidine hydrochloride (Gu.HCl) for longer than 30min exhibited 'clumping' or aggregation making measurements of DNase I activity difficult. Therefore, all measurements were made in the first 30min after addition of the Gu.HCl to the sample. The difference between the total actin content and the actin monomer content is the amount of actin that is polymeric within the cell.

Finally, it is important to lyse cells in the minimum possible volume of SF buffer (1.5×10^7 cells/mL SF buffer was found to be a good ratio). This results in actin concentrations in the

lysate which allow addition of very small volumes of lysate, and Gu.HCl-treated lysate, to the DNase I assay system (5-10uL in 3mL of assay solution) thus diluting the Triton X-100 and Gu.HCl to levels that do not interfere with the assay itself.

The protein concentration in the cell lysates was determined by the method of Lowry *et al.* (1951), using bovine serum albumin as a standard and with the addition of 0.3% (w/v) SDS to overcome the interfering effects of Triton X-100 present in cell lysate samples.

2.3 Results

2.3.1 Assessment of Actin Organization by Fluorescence Microscopy.

The microfilament organization of the cell lines was examined by fluorescence microscopy (Fig. 9) and a summary of the results for all the cell lines examined is presented in table 12. The analysis of these cell lines showed that tumorigenic and non-tumorigenic cells can be distinguished on the basis of microfilament organization.

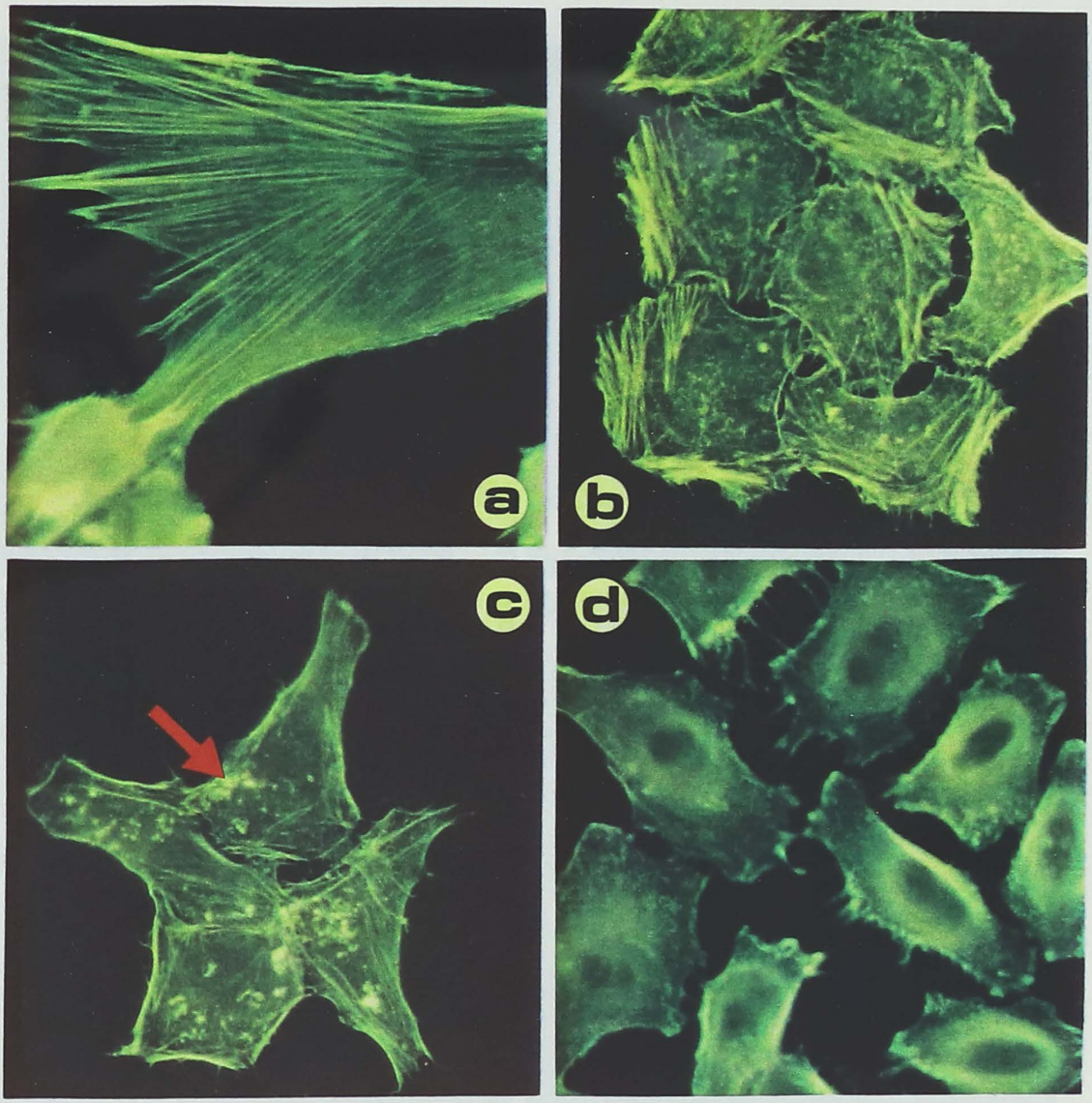


FIGURE 9: A selection of photomicrographs representative of the cell lines studied. The microfilaments have been visualized with NBD-phallacidin. All photographs were taken at a magnification of 200X.

a: MRC-5, normal human fibroblast (nontumorigenic); b&c: D98 AH2, HeLa variant (tumorigenic). The arrow indicates some intensities of fluorescence. d: H.Ep.2, human bronchocarcinoma cell line (tumorigenic).

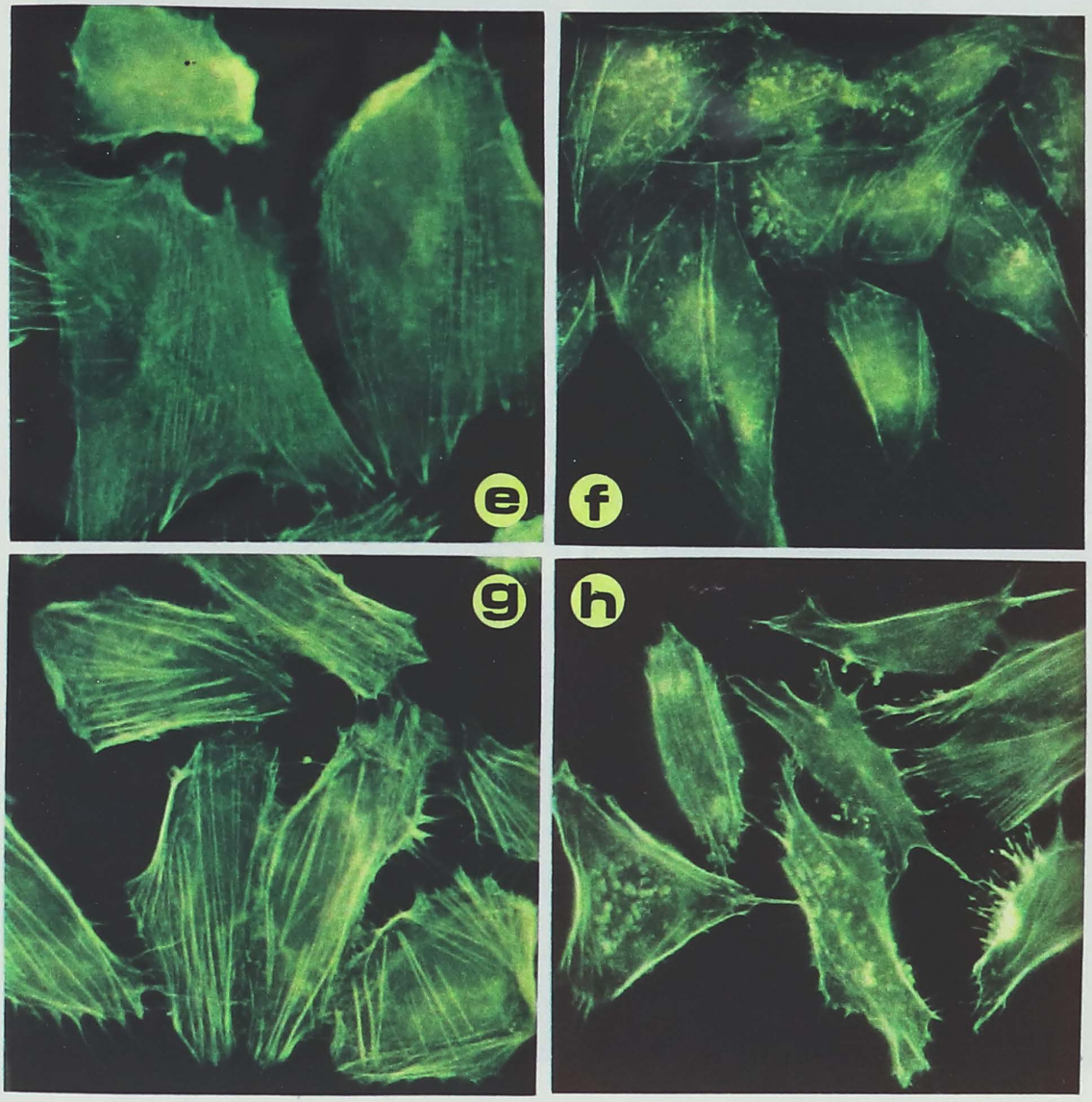


FIGURE 9 (cont.) e: 5E (nontumorigenic); f: 5L (tumorigenic); g: 39E C13 (nontumorigenic); h: ESH 39 (tumorigenic);

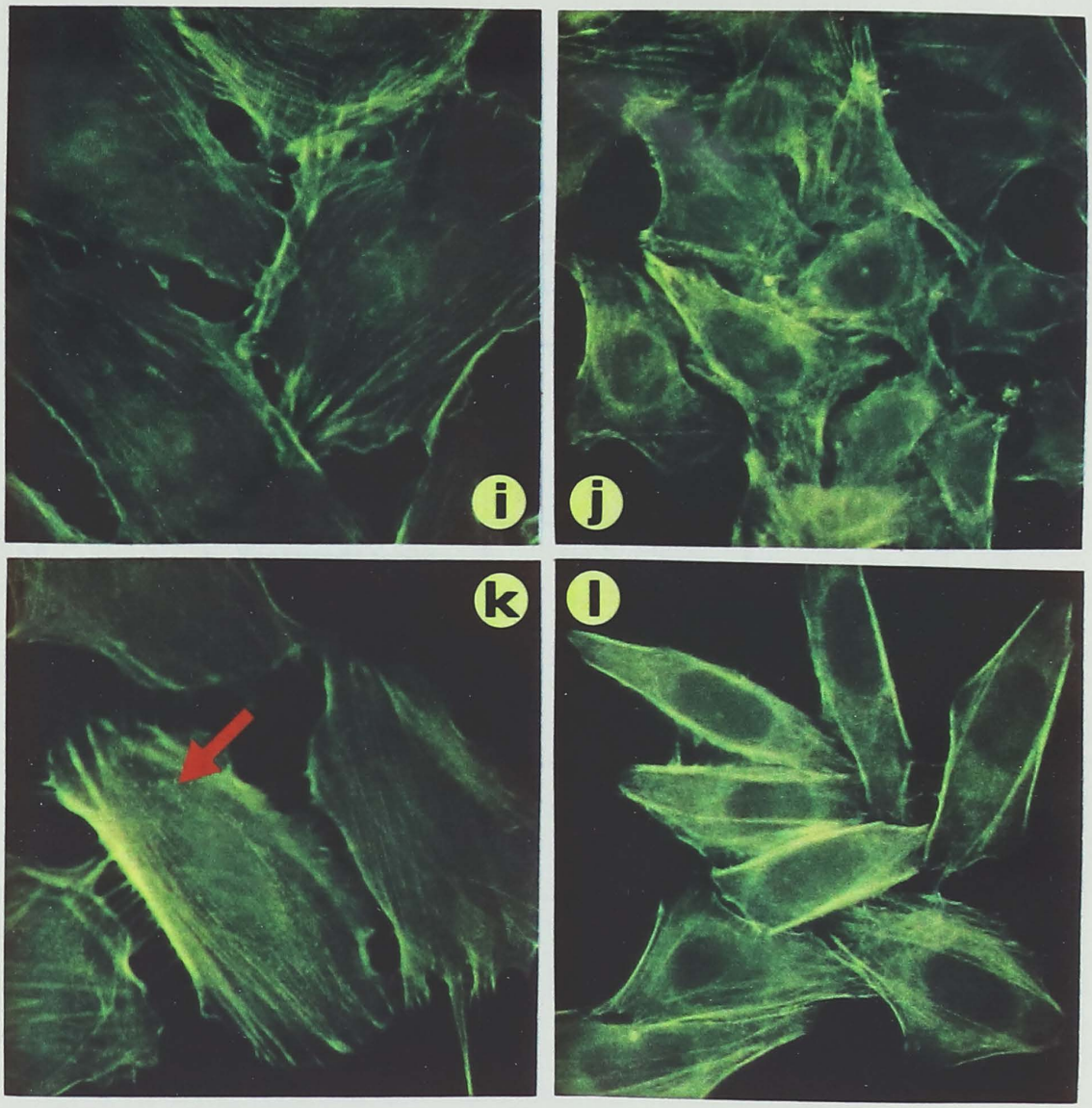


FIGURE 9 (cont.) i: IA3 cn 2.1 (nontumorigenic); j: IA3 cn TG (tumorigenic); k: CN2 Bl Coll (nontumorigenic); The arrow indicates some focal adhesion plaques. l: 5A7 mp 26.15 (tumorigenic).

TABLE 12: A qualitative comparison of the microfilament organization in the cell lines studied.

Cell line	Status	Presence of Stress Fibres	Presence of Intensities of Fluorescence
MRC-5	Nontumorigenic	+++++	-
H.Ep.2	Tumorigenic	-	±
D98 AH2	Tumorigenic	+	+++
5E	Nontumorigenic	++++	-
5L	Tumorigenic	++	++++
39E C13	Nontumorigenic	++++	-
ESH 39	Tumorigenic	++	+++
1A3 cn 2.1	Nontumorigenic	+++	-
IA3 cn TG	Tumorigenic	++	+
CN2 B1 Coll	Nontumorigenic	++++	-
5A7 mp26.15	Tumorigenic	+	+

The diploid fibroblast (MRC-5) cell line is similar to those used by Stanbridge and by Klinger for the production of the somatic cell hybrids that form the basis of this study. The MRC-5 cells displayed well-developed parallel arrays of stress fibres (bundles of microfilaments) running the entire length of the cell (Fig. 9a). These stress fibres were evenly distributed throughout the cytoplasm and obscured the nucleus. The cells were flattened and well spread suggesting strong adhesive properties. At confluency the cells tended to align in parallel without overlapping.

The D98 AH2 cell line is a HeLa variant and was the tumorigenic parent used for the production of the HeLa/fibroblast hybrids. The D98 AH2 cells were small and rounded in shape and varied considerably in microfilament organization (Fig. 9b,c). Stress fibres were present in most of these cells but were never spread over the entire cell and tended to be shorter and apparently finer than those of the MRC-5 cells. Some D98 AH2 cells contain 'intensities' of fluorescence (see arrow in Fig. 9c) that are larger than the focal adhesion plaques. The latter are seen as small points of fluorescence when the focal plane is at the level of the adherent cell surface (see arrow in Fig. 9k).

All the nontumorigenic hybrid cell lines (Fig. 9e,g,i,k) contained well-organized stress fibres which, although not as

numerous as those seen in the MRC-5 cells, were more numerous and more evenly distributed throughout the cells than those of the tumorigenic hybrids (Fig. 9f,h,j,l). No intensities of fluorescence were seen in any of the nontumorigenic cells.

The tumorigenic cells exhibited variable morphologies but tended to be more rounded and appeared smaller than the nontumorigenic cells (all photos were taken at the same magnification). The intensities of fluorescence were found in large numbers in the 5L (Fig. 9f) and ESH 39 (Fig. 9h) cell lines but were present less frequently in the 5A7 mp 26.15 (Fig. 9l) and IA3 cn TG (Fig. 9j) cell lines.

A bronchocarcinoma (H.Ep.2) cell line was also examined (Fig. 9d). These cells were very rounded and no stress fibres could be detected within them. NBD-phallacidin labelling of these cells resulted in intense but diffuse cytoplasmic fluorescence. Some intensities of fluorescence were seen but these may be associated with membrane protrusions rather than being cytoplasmic inclusions.

2.3.2 Analysis of Actin Content by the DNase I-Inhibition Assay.

The actin content of the cell lines is summarized in table 13, which also shows the results of a t-test applied to the data grouped into tumorigenic and nontumorigenic phenotypes. The total actin content is significantly lower in the tumorigenic cell lines with the mean total actin content of the tumorigenic cells being 65% of the nontumorigenic value. This is true whether the data are expressed as the amount of actin per cell or the amount of actin relative to total protein. Despite the difference in total actin content, the tumorigenic and nontumorigenic cell lines were found to contain a remarkably constant ratio of monomeric to total actin (Table 13).

It has been reported that the actin content of cells changes with cell density (Rubin *et al.*, 1978, Blikstad & Carlsson, 1982). The total actin content of HeLa cells was determined by Blikstad & Carlsson (1982) to be 10pg/cell at a density of $3x10^4$cells/cm^2, a figure in close agreement with our measurements of total actin content in D98 AH2 cells (9.0pg/cell at a density of $5x10^4$cells/cm^2). Blikstad & Carlsson (1982) found the total actin content of HeLa cells decreased with increasing cell density in a

TABLE 13: Actin content of lysed cell suspensions as determined by the DNase I-inhibition assay.

Cell Line	Status*	% Monomeric Actin	% Total Actin Per Unit Protein	pg Total Actin per cell
D98 AH2	T	32.8	3.2	9.0
5L	T	32.4	2.7	9.7
ESH 39	T	36.0	3.3	10.8
IA3 cn TG	T	39.5	4.0	9.8
5A7 mp 26.15	T	36.7	3.2	8.2
(Average)		35.5	3.3	9.5
MRC-5	NT	40.3	4.4	17.3
5E	NT	34.1	4.4	16.0
39E C13	NT	37.5	4.7	15.0
IA3 cn 2.1	NT	33.1	5.6	15.1
CN2 B1 Coll	NT	35.2	5.2	11.5
(Average)		36.0	4.9	15.0
t-statistic		-0.304	-5.192	-5.115
Degrees of freedom		8	8	8
		No significant difference	Significantly different with P<0.001	Significantly different with P<0.001

* T = tumorigenic, NT = nontumorigenic.

linear fashion, reaching .5pg/cell at a density of $3x10^5$ cells/cm^2. However, over a smaller range of cell densities (Table 14), the actin content was found to be independent of cell density in both tumorigenic and nontumorigenic cells. Therefore, the differences detected in the total actin content of tumorigenic and nontumorigenic HeLa/fibroblast somatic cell hybrids is not the result of the effect of differing cell densities.

2.4 Discussion

These studies of the HeLa/fibroblast somatic cell hybrids have shown that the tumorigenic cells can be distinguished from the nontumorigenic cells on the basis of microfilament organization and actin content. All of the nontumorigenic cells have microfilaments organized into a large number of stress fibres, evenly distributed throughout the cell and apparently spanning the entire length of the cell. In contrast, the tumorigenic cells have fewer, shorter stress fibres, generally restricted to the edges of the cell, and a lower total actin content (decreased by approximately 35%). In addition, some tumorigenic cells contain intensities of NBD-phallacidin fluorescence. Since NBD-phallacidin associates specifically with

TABLE 14: The effect of cell density on actin content.

Cell line	Density (cells/cm^2)	Total actin/cell (pg)
5E	1.3×10^4	24.3
5E	2.2×10^4	18.2
5E	7.1×10^4	24.7
5L	4.3×10^4	9.7
5L	5.8×10^4	8.9
5L	1.6×10^5	9.5

F-actin (Barak & Yocum, 1981) it is concluded that these observed intensities contain F-actin.

The reduced display of stress fibres is typical of transformed cells derived from fibroblasts or other cells of mesenchymal origin (Brinkley, 1982) but this general observation cannot be extended to all cell types. Watt *et al.* (1978), on the basis of a comparison of tumorigenic and nontumorigenic mouse hybrid pairs, concluded that, while hybrid cell pairs derived from the PG19/diploid mouse fibroblast fusion did show an association of disorganized microfilaments with tumorigenicity, such an association was not a constant feature of all the cell types studied. Bannikov *et al.* (1982) in studying a series of cell lines derived from rat liver found that although alterations in the ability of cells to spread on the substratum and to form cell-cell contacts were common features of morphologically transformed fibroblastic and epithelial cell cultures, the accompanying changes in cytoskeletal structures were different in various cell types. The cell lines examined in this study were derived from a single tumour cell line (the HeLa variant D98 AH2) and normal fibroblast cell lines. The consistent loss of microfilament organization in the tumorigenic hybrids observed in this study could reflect the nature of the fibroblasts used to produce them or be a characteristic of human epithelial tumour hybrids. While there is some evidence for alterations of microfilament organization in transformed cells of epithelial

origin (Keski-Oja *et al.*, 1983), further study will be required to characterize such changes in non-fibroblastic cell types.

Most cells *in vivo* do not contain stress fibres, although it is difficult to assess microfilament organization in intact tissue. It has therefore been suggested that stress fibres are induced by the unique conditions of *in vitro* tissue culture and, as such, are not an appropriate indicator of malignant transformation. However, stress fibres do form *in vivo* under certain conditions. The endothelial cells lining the aorta, and other major blood vessels, exhibit stress fibres aligned in the direction of blood flow (Drenckhahn & Wagner, 1986). Following deliberate injury to the lens or corneal epithelium, stress fibres are seen in the cells that migrate into the wound area (Gordon *et al.*, 1982). It can be concluded that, under appropriate environmental conditions, the formation of stress fibres will occur *in vivo*. Presumably the conditions provided by the tissue culture environment are an example of a situation which will induce stress fibre formation, at least in fibroblasts. Under these conditions the pattern of stress fibre formation is a useful and easily visualized marker of microfilament organization. The inability of tumorigenic cells to form stress fibres under conditions which result in the formation of large numbers of stress fibres in nontumorigenic cells, must indicate a differing ability to respond to those environmental conditions. It is therefore of interest to investigate why tumorigenic cells are

unable to organize their microfilament system in response to the same conditions.

Cellular adhesion, morphology and microfilament organization are closely inter-related functions and it has been suggested that the loss of microfilament organization is a function of cellular adhesiveness and morphology, and not the tumorigenic potential of the cells (Brinkley, 1982). Levels of fibronectin, an extracellular glycoprotein involved in cellular adhesion, are reduced in transformed cells and fibronectin may be associated with the cytoplasmic microfilament network through a transmembrane interaction. This hypothesis is supported by evidence that (1) extracellular fibronectin fibrils are frequently coincident with intracellular microfilament bundles, (2) the treatment of cells with trypsin to remove extracellular fibronectin disrupts intracellular microfilament organization, (3) the disruption of microfilaments with the drug cytochalasin B induces the release of surface fibronectin (Hynes *et al.*, 1981), and (4) that integral membrane proteins have been isolated which bind to extracellular fibronectin and to intracellular cytoskeletal proteins (Tamkun *et al.*, 1986).

The addition of exogenous fibronectin, or agents that raise the intracellular levels of cAMP, to transformed cells causes the cells to become more adherent and assume a morphology resembling that of normal fibroblasts. This alteration in morphology is

accompanied by the reorganization of microfilaments and the whole process has been termed "reverse transformation" (Yamada *et al.*, 1976; Ali *et al.*, 1977; Puck, 1977; Pastan *et al.*, 1982; Leader *et al.*, 1983). The reappearance of bundles of microfilaments during a reorganization of the microfilament system in association with a more flattened morphology as part of the reverse transformation process has been taken to indicate that microfilament complexity is related to cellular morphology and not to tumorigenic potential (Willingham *et al.*, 1977; Der *et al.*, 1981). However, the state of reverse transformation is not stable; upon removal of the agent inducing the reverse transformation, the cells revert to the original rounded morphology and disorganized cytoskeleton. Consequently, it is not possible to assess the tumorigenic potential of the reverse transformed cells.

One of the reasons for selecting the HeLa/fibroblast somatic cell hybrid system for this study was that the tumorigenic and nontumorigenic cells have very similar morphologies and adhesive properties. It is, therefore, unlikely that the differences in microfilament organization in these cells is attributable to morphological differences.

A possible association between microfilament disorganization and metastatic potential has been suggested, initially on the grounds that metastasis requires the cells to detach from the substratum and to actively migrate through surrounding tissues,

processes in which the microfilament system may be involved (Raz & Geiger, 1982). Studies of cells of varying metastatic abilities gives some support to this hypothesis. Volk *et al.* (1984) found in 14 of 15 clones of the murine K-1735 melanoma tumour that the degree of actin organization was inversely correlated with metastatic ability, with the one exception possessing a highly organized microfilament system and high metastatic potential. A similar inverse correlation between metastatic capability and actin organization has been reported for rat adenocarcinoma cells (Zachary *et al.*, 1986). However, studies carried out by Friedman *et al.* (1985) suggest that any relationship between microfilament organization and metastatic potential may be more complex than a simple inverse correlation. Hereditary adenomatosis of the colon and rectum is a disorder characterized by the development of numerous colonic polyps (benign adenomas) and subsequently carcinoma of the large bowel. Apparently normal epithelial cells from patients with hereditary adenomatosis, when grown *in vitro* exhibit a loss of actin organization in comparison with normal colonic epithelial cells. The ability to organize actin into stress fibres is regained in cells from benign adenomas while cells from the malignant adenocarcinomas exhibited the least number of stress fibres (Friedman *et al.*, 1985).

Both Volk *et al.* (1984) and Di Renzo (1985) observed that clones with a low metastatic ability were able to react with and organize extracellular fibronectin into fibres but clones with a

high metastatic ability, while able to interact with extracellular fibronectin, were unable to organize the fibronectin into fibres. Cultured skin fibroblasts from patients with hereditary adenomatosis exhibit a disorganized microfilament system. In these cells both fibronectin synthesis and binding to the cell surface were normal and the addition of exogenous fibronectin did not induce the appearance of an organized microfilament system (Kopelovich *et al.*, 1985). The defect in these cells may lie in the transmembrane association between actin and fibronectin.

With the HeLa/fibroblast hybrid cell system, tumorigenicity is assessed by the injection of cells into nude (athymic) mice. In nude mice metastasis does not occur, at least within the testing period, and hence the metastatic potential of the hybrid cells cannot be assessed. Metastasis itself involves several stages and to attempt to relate microfilament organization to metastatic potential and to tumorigenicity would result in an overly complex study. Treatment of the tumorigenic HeLa/fibroblast somatic cell hybrids with sodium butyrate or dexamethasone results in a reverse transformation causing the cells to resemble their nontumorigenic counterparts. In addition to the acquisition of a flattened morphology, this reverse transformation is associated with the organization of microfilaments into bundles and of fibronectin into fibrils (Der *et al.*, 1981). Thus, in these cells, if the transmembrane association between actin and fibronectin is affected in the

tumorigenic cells, this defect must be corrected, at least in part, by the reverse transformation.

The intensities of NBD-phallacidin fluorescence were observed only in the tumorigenic HeLa/fibroblast hybrids, and must represent some sort of aggregate of F-actin. The presence of these aggregates in tumorigenic cells may reflect the inability of the tumorigenic cells to maintain highly organized stress fibres. Carley *et al.* (1981), using NBD-phallacidin to visualize the microfilaments, have previously reported the presence of F-actin aggregates near the ventral surface of cells transformed by RNA or DNA tumour viruses, by chemical mutagens or spontaneously. These F-actin aggregates contain the actin-binding proteins alpha-actinin and fimbrin but not tropomyosin (Carley *et al.*, 1985). The absence of tropomyosin, a protein which stabilizes F-actin structures, indicates that these aggregates are potentially of a dynamic nature. Transformed cells have reduced levels of tropomyosin, with levels of the higher molecular weight isoforms being selectively lost. The F-actin aggregates may be abnormal structures formed as a consequence of reduced levels of tropomyosin. However, the formation of such actin patches occurs in normal rat kidney cells transformed by a temperature-sensitive mutant of RSV within 30min of a shift to the permissive temperature (Carley *et al.*, 1981). This would suggest a mechanism other than depletion of tropomyosin to be important in the formation of these F-actin aggregates.

Boschek *et al.* (1981) and Marchisio *et al.* (1984) describe actin-containing 'ruffles' or actin 'flowers' which may be similar structures to the F-actin aggregates. These structures or 'ruffles' appear on the dorsal surface of cells transformed by temperature-sensitive mutants of RSV as early as 15min after temperature shift, stain intensely with fluorescent derivatives of phalloidin, contain, in addition to actin, the actin-binding proteins alpha-actinin, myosin and tropomyosin, and seem to correlate with membrane protrusions (Boschek *et al.*, 1981). Although these structures appear within 15-20min of the temperature shift, loss of stress fibres and rounding of the cells do not occur until 6 to 12 hours after temperature shift (Boschek *et al.*, 1981). In RSV-transformed cells these structures may represent an early stage of microfilament reorganization, possibly as a result of the disruption of the normal interactions between the microfilaments and the plasma membrane. A number of proteins, including vinculin, metavinculin and talin, have been suggested to be involved in the attachment of microfilaments to the plasma membrane. Alterations in any of these proteins, e.g. by phosphorylation, might disrupt the normal interactions between microfilaments and the membrane thereby preventing the formation of stable stress fibres. Indeed, vinculin from RSV-transformed cells has been shown to contain increased amounts of phosphotyrosine (Sefton *et al.*, 1981) and both vinculin and talin may be phosphorylated by protein kinase C, the proposed receptor

for the phorbol ester tumour promoters (Werth & Pastan, 1984; Litchfield & Ball, 1986).

The total actin content of the tumorigenic HeLa/fibroblast hybrid cells was significantly lower than that of the nontumorigenic cells. Actin content has been examined in relation to malignant transformation in other systems and, while the results are variable, in most cases actin content has been found to be either unchanged or decreased in malignant cells. The actin content of lymphocytes from subjects with chronic lymphocytic leukaemia has been found to be less than that of normal lymphocytes (Atkins & Anderson, 1982; Stark *et al.*, 1982) with the magnitude of reduction (35%) being similar to that found in the HeLa/fibroblast hybrid system. A reduced actin content has also been noted in transformed cell lines of lymphoid and myeloid origin in a comparison with normal lymphocytes and leukocytes (Varani *et al.*, 1983). Atkins & Anderson (1982) in addition found the tubulin content of leukaemic lymphocytes to be significantly less (by 33%) than that of normal lymphocytes. However, the comparison of non-adherent white blood cells with the adherent HeLa/fibroblast hybrid cells should be made with caution, and in SV40-transformed 3T3 fibroblasts, compared with nontransformed 3T3 cells, a significant decrease in tubulin content, but not actin content, was observed (Fine & Taylor, 1976).

The variability in measurements of actin content may be the

result of the different methods used. The most common method is SDS-polyacrylamide gel electrophoresis followed by densitometry or determination of levels of a radioactive tracer in an excised band, to quantitate the amount of protein(s) of interest. The DNase I-inhibition assay in comparison, requires less manipulation of the sample and, provided appropriate methods of cell lysis and buffering are used in conjunction with standard curves prepared from highly pure native actin monomer, total actin levels and the proportion of the actin pool present as monomer can be accurately determined.

The reduced total actin content of tumorigenic cells could be due to specific suppression of actin synthesis, an increase in the rate of actin degradation or the redistribution of actin into a pool not measurable by the techniques used in these experiments. Koffer *et al.* (1983) suggest that as much as 15% of the total cellular actin remains inaccessible to the DNase I-inhibition assay, even after exposure to a depolymerizing medium but these data were obtained after the use of nitrogen cavitation to lyse the cells and centrifugation to concentrate membranous material into one fraction. Such a technique would be expected to leave membrane fragments and vesicles which might trap actin in an environment inaccessible to DNase I. In comparison, the method used in this study utilized Triton X-100 to lyse the cells and disperse membrane lipids prior to assay. While a portion of the cellular actin might remain inaccessible to assay by DNase

I-inhibition, it is unlikely to account for the 35% reduction in actin content detected in the tumorigenic cells.

The 30-40% reduction in total actin content of the tumorigenic somatic cell hybrids is unlikely, on its own, to have a marked effect on the degree of actin polymerization, since the actin concentrations within the cell (>30uM) should still be substantially greater than the critical actin concentration required for polymerization (0.4uM; Korn, 1982). Despite the reduction in total actin content in the tumorigenic cells, the proportion of the total actin present as actin monomer was constant in all the cell lines (36%). This level of monomeric actin (>12 uM) is well above the critical concentration for actin polymerization and therefore most of the monomeric actin pool is probably present as a complex with other proteins, such as profilin. The observation that approximately one third of the total cellular actin is maintained in the form of actin monomer suggests that the existence of this pool is important to normal cellular function, possibly to allow the induction of rapid polymerization under the influence of certain stimuli. Presumably, the size of the monomer pool is controlled by some sort of regulatory mechanism, possibly the coordinate synthesis of profilin and actin or the action of appropriate capping proteins.

The reduction in total actin content could occur either by a balanced loss of actin isoforms or by a selective loss of

particular isoforms since the DNase I-inhibition assay is not isoform specific. In normal fibroblasts alpha-actin is abundantly coexpressed with the beta and gamma forms but in transformed fibroblasts levels of alpha-actin are reduced, apparently as a result of the specific suppression of alpha-actin synthesis (Witt *et al.*, 1983; Leavitt *et al.*, 1985). The stimulation of quiescent AKR-2B mouse embryo cells with EGF results in a rapid and specific induction of mRNAs coding for beta- and gamma- but not alpha-actin (Elder *et al.*, 1984). However, while physiological changes in cells may be correlated with alterations in isoform ratio, the significance of the ratio of actin isoforms to microfilament organization is uncertain since no major distinction between function or location of the isoforms has yet been made.

The DNase I-inhibition assay distinguishes actin monomer from polymeric actin but it does not distinguish different degrees of F-actin organization. Since the ratio of monomeric to total actin was constant in all the cell lines while total actin content was decreased, the tumorigenic cells must contain reduced levels of F-actin. In addition, the F-actin pool of tumorigenic cells may differ from that of nontumorigenic cells in mean filament length or degree of crosslinking. The mean filament length and degree of crosslinking, as well as the interactions of microfilaments with other cellular structures, are controlled by the various actin regulatory proteins present in nonmuscle cells. The alteration in microfilament organization associated with the re-expression of

tumorigenicity is likely to be caused by alterations in the activity of these regulatory proteins and not by alterations in actin itself for several reasons. The most compelling evidence that the actin molecule is unaltered in tumorigenic cells comes from reverse transformation of the tumorigenic cells by sodium butyrate or dexamethasone (Der et al., 1981) which demonstrates that, under certain conditions, the actin of tumorigenic cells will form stable polymeric structures and is therefore functionally competent. In addition, actin from the tumorigenic cells exhibits the same mobility in polyacrylamide gels as does actin from the nontumorigenic cells and rabbit skeletal muscle actin. Further, actin purified from lymphocytes from normal and chronic lymphocytic leukaemia subjects was found to exhibit identical polymerization properties, although the kinetics of actin-activated myosin ATPase activity was found to differ (Liebes et al., 1983).

Alterations in the activity of the actin regulatory proteins could lead to alterations of microfilament organization. Instability of F-actin structures could lead to an increased turnover of actin polymer with the actin monomer pool and since degradation of actin probably occurs through the more susceptible monomer pool, this could lead to an increased rate of actin degradation and, consequently, reduced actin levels in the cell. Alternatively the reduced levels of total actin in the cell may result from the specific suppression of actin synthesis.

2.5 Conclusions

In the HeLa/fibroblast somatic cell hybrid system, the re-expression of tumorigenicity is associated with an alteration in microfilament organization and a reduced total actin content. These changes are unlikely to reflect differing morphologies and adhesive properties as the tumorigenic and nontumorigenic hybrid cell pairs are morphologically very similar.

While it is possible that the altered pattern of microfilament organization results from the reduced actin content of the tumorigenic cells, it is more likely that the altered organization results from changes in the actin-binding proteins which regulate the organization and interactions of actin.

The reduction in total actin content may result from the specific suppression of actin synthesis or an increase in actin degradation. An increased rate of actin degradation may be the result of the instability of F-actin structures.

Chapter 3

ACTIN SYNTHESIS IN TUMORIGENIC AND NONTUMORIGENIC HUMAN HYBRID CELLS.

3.1 Introduction

In the HeLa/fibroblast hybrid cell system the re-expression of tumorigenicity is associated with altered microfilament organization, notably the loss of actin stress fibres, and a significant reduction in the total actin content. The reduced total actin content in tumorigenic cells could be due to specific suppression of actin synthesis, an increase in the rate of actin degradation or the redistribution of actin into a pool not measurable by the techniques used.

A suppression of actin synthesis associated with malignant transformation has been detected in other cellular systems. Leavitt *et al.* (1980) found that normal T lymphocytes synthesize an abundant amount of beta-actin but relatively little gamma-actin. In comparison, the Molt-4 cell line of leukaemic lymphocytes synthesize apparently equal amounts of beta- and gamma-actin. At the same time actin decreased from 16% of total protein in normal T lymphocytes to 6% in Molt-4 cells. However, Leavitt *et al.* (1980) concluded that this reduced total actin content is due to the relative enhancement in synthesis of other highly abundant polypeptides. In NIH3T3 (mouse) and Rat-2

fibroblasts, alpha-actin is abundantly coexpressed with beta-and gamma-actin but following transformation by chemical carcinogens or oncogenic DNA, the synthesis of alpha-actin is suppressed (Leavitt *et al.*, 1985). A reduction in the amount of alpha-actin has also been observed in RSV-transformed chick embryo fibroblasts. The use of a temperature-sensitive mutant of RSV demonstrated this reduction to be reversible and transformation-related. It was concluded that the reduction in the amount of alpha-actin was due to decreased synthesis rather than an increase in degradation (Witt *et al.*, 1983). In view of these findings, the studies described in this chapter aim to provide a comparison of actin synthesis in the tumorigenic and nontumorigenic HeLa/fibroblast hybrid cells and thus determine whether the reduction of the total actin content in the tumorigenic cells is due to the suppression of actin synthesis.

The accurate determination of the rate of protein synthesis, by the incorporation of radioactive amino acids, requires the determination of the specific radioactivity of the amino acid, both in the protein molecule and in the precursor pool (Zak *et al.*, 1979; Rannels *et al.*, 1982). This requirement can be satisfied by techniques such as assessment of fractional rates, in which the specific radioactivity of a pulse label is determined by comparison with an equilibrium label which is at uniform specific radioactivity in all cellular compartments (Clark & Zak, 1981). However, these techniques are unmanageable for the comparison of

actin synthesis in a number of cell lines.

The method developed and utilized for this study is rapid, simple, reproducible and provides a comparative measurement of actin synthesis in the tumorigenic and nontumorigenic cell lines. The procedure again relies on the high affinity and specificity with which actin monomer binds to the enzyme deoxyribonuclease I (DNase I). Actin can be rapidly extracted from cell lysates, appropriately treated to ensure greater than 90% of the total cellular actin is in a state of native actin monomer, by adsorption onto DNase I covalently linked to Sepharose. By comparing the incorporation of a radioactive amino acid into DNase I-Sepharose-bound material with the incorporation of radioactivity into total cellular protein, a measurement of actin synthesis relative to total protein synthesis can be obtained. While the technique does not allow determination of the absolute rate of actin synthesis, the measurement of actin synthesis relative to total protein synthesis is not affected by variations in specific radioactivity of the precursor pool or differences in cell size or growth rate.

3.2 Materials and Methods.

L-[^{35}S]methionine (1160 Ci/mmol, 15mCi/mL) was obtained from Amersham International plc. (Code SJ.235). Minimum essential medium eagle (modified) without glutamine and methionine (referred to as methionine free medium) was purchased from Flow Laboratories (Cat. No. 16-222-49). Cell culture techniques were described in Section 2.2.1.

3.2.1 Preparation of DNase I-linked Sepharose-4B

Freeze-dried CNBr-activated Sepharose 4B (from Pharmacia (Australia) Pty. Ltd.) was reswollen with 1mM HCl (200mL/g Sepharose). Deoxyribonuclease I (EC 3.1.21.1 from bovine pancreas, lyophilized, from Sigma Chemical Co., St. Louis, U.S.A.) was dissolved in $NaHCO_3$ buffer (0.1M $NaHCO_3$, pH 8.3, containing 0.5M NaCl). This protein solution was mixed with the Sepharose (15-20mg DNase I per gram Sepharose, dry weight) for 2h at room temperature. The remaining active groups on the Sepharose were blocked with 0.2M glycine for 2h at room temperature. Excess

adsorbed protein was removed by washing the Sepharose with $NaHCO_3$ buffer, followed by acetate buffer (0.1M sodium acetate, pH 4, 0.5M NaCl) followed by $NaHCO_3$ buffer. The DNase I-Sepharose conjugate was stored at 4°C in G-buffer (200uM ATP, 200uM $CaCl_2$, 2mM Tris.HCl, pH 8.0, 1.5mM NaN_3).

3.2.2 Measurement of Relative Actin Synthesis

Cells were seeded onto 1.2cm diameter glass coverslips at a density of $1x10^4$ cells/cm^2 in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% (v/v) foetal calf serum (FCS), 5% (v/v) newborn calf serum (NCS), 2mM pyruvate and antibiotics (50 units/mL penicillin G, 50 units/mL streptomycin sulphate and 180 units/mL neomycin sulphate). In the case of the MRC-5 cell line, 10% (v/v) FCS was used.

After incubation for 48h at 37°C in a 5% CO_2 humidified atmosphere, the coverslips were rinsed twice with phosphate buffered saline (PBS: 7mM Na_2HPO_4, 3mM NaH_2PO_4, 137mM NaCl) and transferred to a 24-well sterile plastic multi-dish. To each coverslip was added 0.5mL of methionine-free medium supplemented with 10% (v/v) dialysed FCS, 2mM pyruvate, antibiotics (as above) and 2mM glutamine and containing 3uM unlabelled methionine and

[^{35}S]methionine (approx. 70uCi per 10mL medium). The cells were incubated at 37^{o}C in this medium for various times and at the end of the incubation period the multi-dish was placed on ice, the [^{35}S]methionine medium aspirated and each coverslip rinsed twice with 1mL volumes of ice-cold PBS. DMEM (1mL/well) supplemented with 5% (v/v) FCS, 5% (v/v) NCS and 1ug/mL of cycloheximide, was added to each well and the cells were then incubated for 10min at 37^{o}C. The aim of this step was to stop protein synthesis and to 'chase out' the intracellular pool of [^{35}S]methionine. The exchange of extracellular amino acids with intracellular methionine occurs within 5min (Johnstone and Scholefield, 1961).

After this 'cold chase' step the coverslips were rinsed in cold PBS (2x100mL) and transferred to a clean multi-dish on ice. All subsequent procedures were carried out at 4^{o}C. G-buffer (200uM ATP, 200uM $CaCl_2$, 2mM Tris.HCl, pH 8.0, 1.5mM NaN_3) supplemented with 0.3% (w/v) Triton X-100, 0.75M guanidine hydrochloride (Gu.HCl), 0.5mM phenylmethylsulphonylfluoride (PMSF) and 0.1uM leupeptin was added (250uL/coverslip) and then the tray was left for 15min with occasional shaking. The Triton X-100 was added to lyse the cells, the Gu.HCl to convert the polymeric actin to monomer and G-buffer was used to maintain the actin in a native and monomeric form. These conditions cause greater than 90% of total cellular actin to be converted to native monomer as determined by the DNase I-inhibition assay.

The lysates were then transferred to microfuge tubes and the coverslips rinsed with G-buffer (2x250uL) containing 5ug/mL bovine serum albumin (BSA) and the rinsings were then combined with the lysates. The total volume of each lysate was calculated by weight. The radioactivity in two 50uL samples of each lysate was counted to obtain a measure of [^{35}S]methionine incorporation into total protein. Less than 5% of these total counts were not precipitable with 10% trichloracetic acid indicating that nearly all of the methionine was incorporated into protein. The remaining lysate was spun for 10s in a microfuge to pellet the nuclear material. The supernatant was transferred to a microfuge tube and the pellet resuspended in 300uL of G-buffer containing 5ug/mL BSA and again spun in a microfuge for 10s. The supernatants were combined and the pellets discarded.

Two coverslips were used for each incubation time: one becoming a test and the other a control (Fig. 10). DNase I-linked Sepharose 4B, prepared as described in section 3.2.1, was added (100uL, 50% packed volume in G-buffer) to each test lysate and uncoupled Sepharose 4B was added to each control lysate. These lysate/Sepharose mixtures were incubated for 30min with constant tumbling and then the Sepharose was pelleted and the supernatants aspirated. The Sepharose was washed (3x500uL) with high ionic strength wash buffer (0.6M NaCl, 20mM Tris.HCl, pH 7.8, 2mM $CaCl_2$, 1mM dithiothreitol (DTT), 1mM PMSF, 0.1uM leupeptin) to remove non-specifically bound material. For each wash step the Sepharose

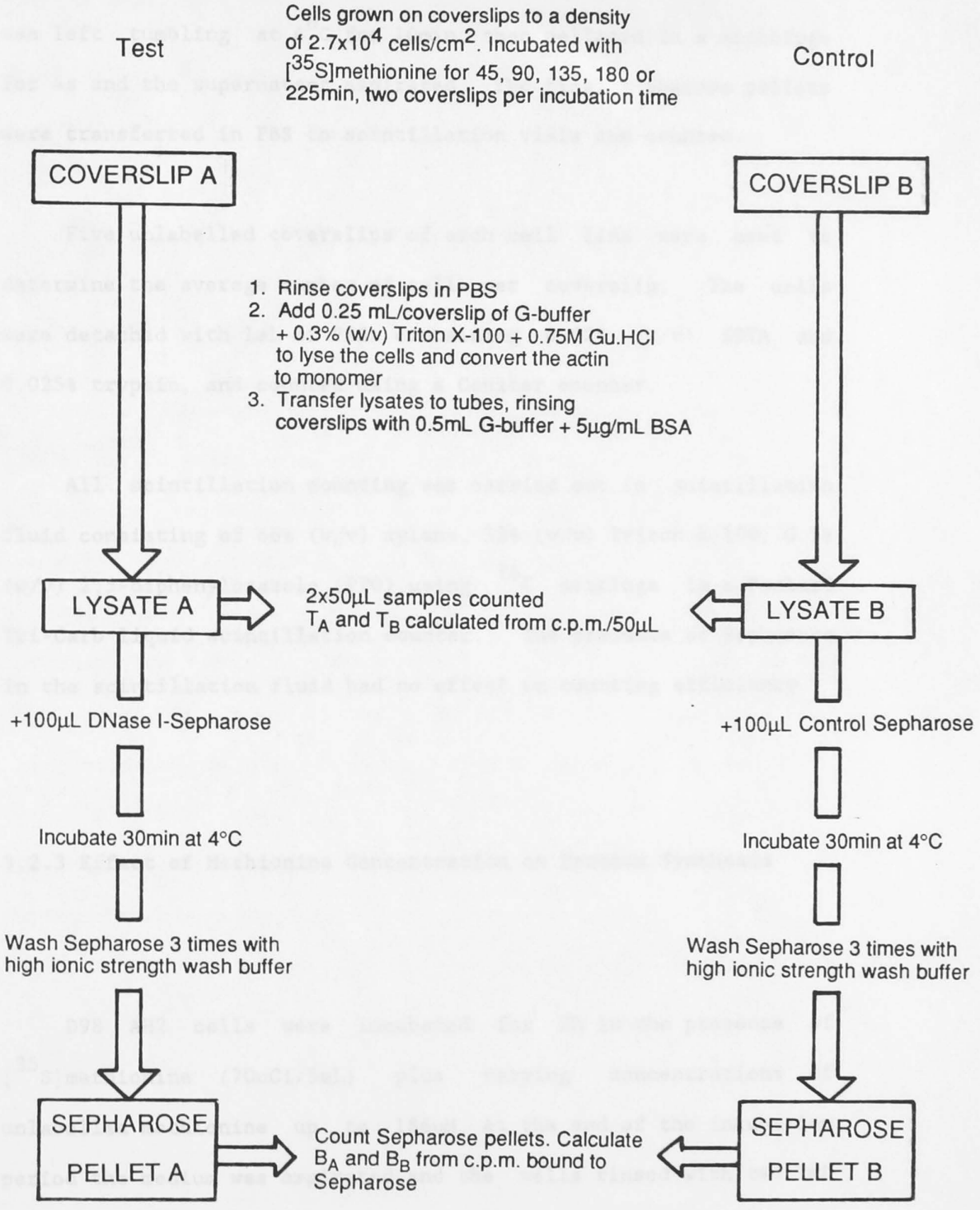


FIGURE 10: Outline of the procedure used to determine the synthesis of actin relative to total protein. T_A and T_B are the incorporation of [^{35}S]methionine into total protein. B_A and B_B are the incorporation of [^{35}S]methionine into Sepharose-bound material.

was left tumbling at 4°C for 10min, then pelleted in a microfuge for 4s and the supernatant aspirated. The final Sepharose pellets were transferred in PBS to scintillation vials and counted.

Five unlabelled coverslips of each cell line were used to determine the average number of cells per coverslip. The cells were detached with 1mL of PBS containing 0.02% (w/v) EDTA and 0.025% trypsin, and counted using a Coulter counter.

All scintillation counting was carried out in scintillation fluid consisting of 66% (v/v) xylene, 33% (v/v) Triton X-100, 0.5% (w/v) 2,5-diphenyloxazole (PPO) using ^{14}C settings in a Packard Tri-Carb liquid scintillation counter. The presence of Sepharose in the scintillation fluid had no effect on counting efficiency.

3.2.3 Effect of Methionine Concentration on Protein Synthesis

D98 AH2 cells were incubated for 2h in the presence of [^{35}S]methionine (70uCi/5mL) plus varying concentrations of unlabelled methionine up to 186uM. At the end of the incubation period the medium was aspirated and the wells rinsed with two 1mL volumes of PBS. The cells were then lysed with G-buffer containing 0.3% (w/v) Triton X-100, 0.75M Gu.HCl, 0.5mM PMSF and 0.1uM

leupeptin, and samples of the lysate counted to determine the incorporated radioactivity.

3.2.4 Specificity of Actin Removal by DNase I-Sepharose

Cells (5L, $6x10^5$) were labelled by overnight incubation with 70uCi of ^{35}S-methionine and were taken through the cell lysis/actin monomerization and Sepharose-binding steps as described above. A sample of each fraction produced by the assay procedure was then dialysed for 48h against 10mM ammonium carbonate (pH 7.5) to remove Gu.HCl, lyophilized, solubilized in 100uL of electrophoresis sample buffer (10% (w/v) glycerol, 0.1% (w/v) bromophenol blue, 2% (w/v) sodium dodecyl sulphate (SDS), 10mM Tris.HCl, pH 8.0, 0.1M DTT) and analyzed by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) and subsequent autoradiography.

See Addendum Point 1

3.3 Results

3.3.1 The Validity of the Assay

It is necessary to establish that the methionine concentration used for the subsequent studies is not rate-limiting for protein synthesis. This is particularly important given that HeLa cells, like many tumorigenic cells, lack a functional pathway for the synthesis of methionine from homocysteine and are therefore dependent on exogenous methionine for growth (Hoffman, 1984). Figure 11 shows the effect of total methionine concentration on the rate of protein synthesis. Increasing the methionine concentration has a biphasic effect; with concentrations of methionine up to approximately 3uM the rate of protein synthesis increased rapidly, while concentrations above 3uM caused protein synthesis to increase only slowly. A methionine concentration of 3uM (approx. 10nM [^{35}S]methionine and 3uM unlabelled methionine) was chosen for subsequent experiments since this gave the highest specific activity without severely compromising the rate of protein synthesis. This concentration of methionine is greater than the published values for the K_m of methionine transport into pigeon erythrocytes (0.06-0.10uM;

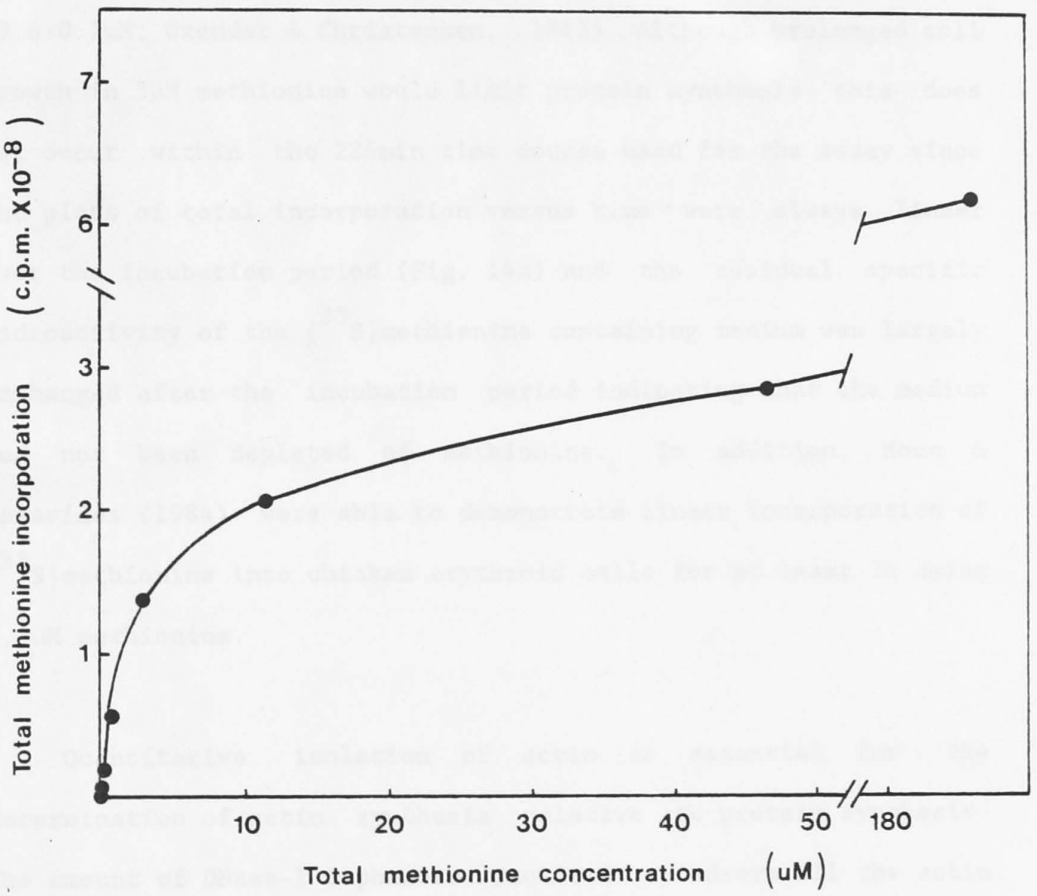


FIGURE 11: Effect of methionine concentration on the rate of protein synthesis.

Cells were incubated for 2h at 37°C in the presence of 70uCi of [^{35}S]methionine and up to 186uM unlabelled methionine, as described in section 3.2.3. Total methionine incorporation was calculated from the ratio of labelled to unlabelled methionine.

Eavenson & Christensen, 1967) and Ehrlich Ascites cells (0.6-0.7uM; Oxender & Christensen, 1963). Although prolonged cell growth in 3uM methionine would limit protein synthesis this does not occur within the 225min time course used for the assay since the plots of total incorporation *versus* time were always linear over the incubation period (Fig. 14a) and the residual specific radioactivity of the [^{35}S]methionine containing medium was largely unchanged after the incubation period indicating that the medium had not been depleted of methionine. In addition, Moon & Lazarides (1984) were able to demonstrate linear incorporation of [^{35}S]methionine into chicken erythroid cells for at least 3h using 0.3uM methionine.

Quantitative isolation of actin is essential for the determination of actin synthesis relative to protein synthesis. The amount of DNase-I Sepharose required to adsorb all the actin from the lysates was determined by titrating the amount of DNase I-Sepharose against cell number and determining the amount of actin not adsorbed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). In addition, it was necessary to determine the optimal concentration of Gu.HCl to ensure maximum conversion to monomer without inhibiting adsorption of the actin monomer to DNase I-Sepharose. D98 AH2 cells ($2x10^5$) were labelled by overnight incubation with 70uCi of [^{35}S]methionine and taken through the cell lysis/actin monomerization procedure. Adsorption of these lysates to DNase I-Sepharose or to control Sepharose was carried

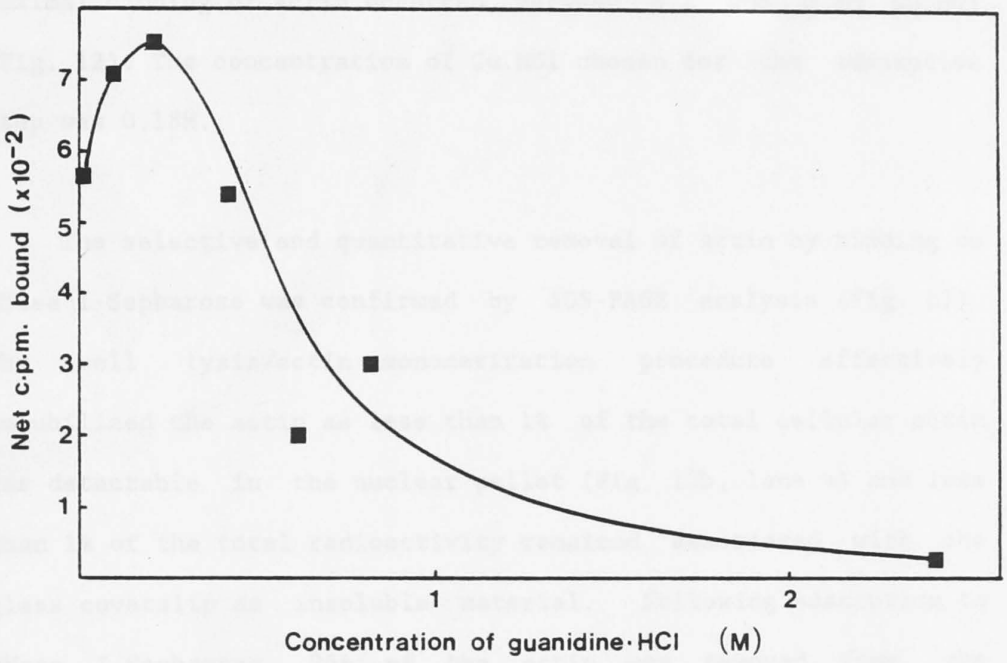


FIGURE 12: Effect of guanidine hydrochloride concentration on the adsorption of actin to DNase I-Sepharose.

D98 AH2 cells, labelled by overnight incubation in the presence of 70uCi of [^{35}S]methionine, were taken through the cell lysis/actin monomerization procedure. Adsorption to DNase I-linked or control Sepharose was carried out in the presence of a range of guanidine hydrochloride concentrations up to 3M. Net c.p.m bound was calculated by subtracting c.p.m. bound to control Sepharose from c.p.m. bound to DNase I-Sepharose.

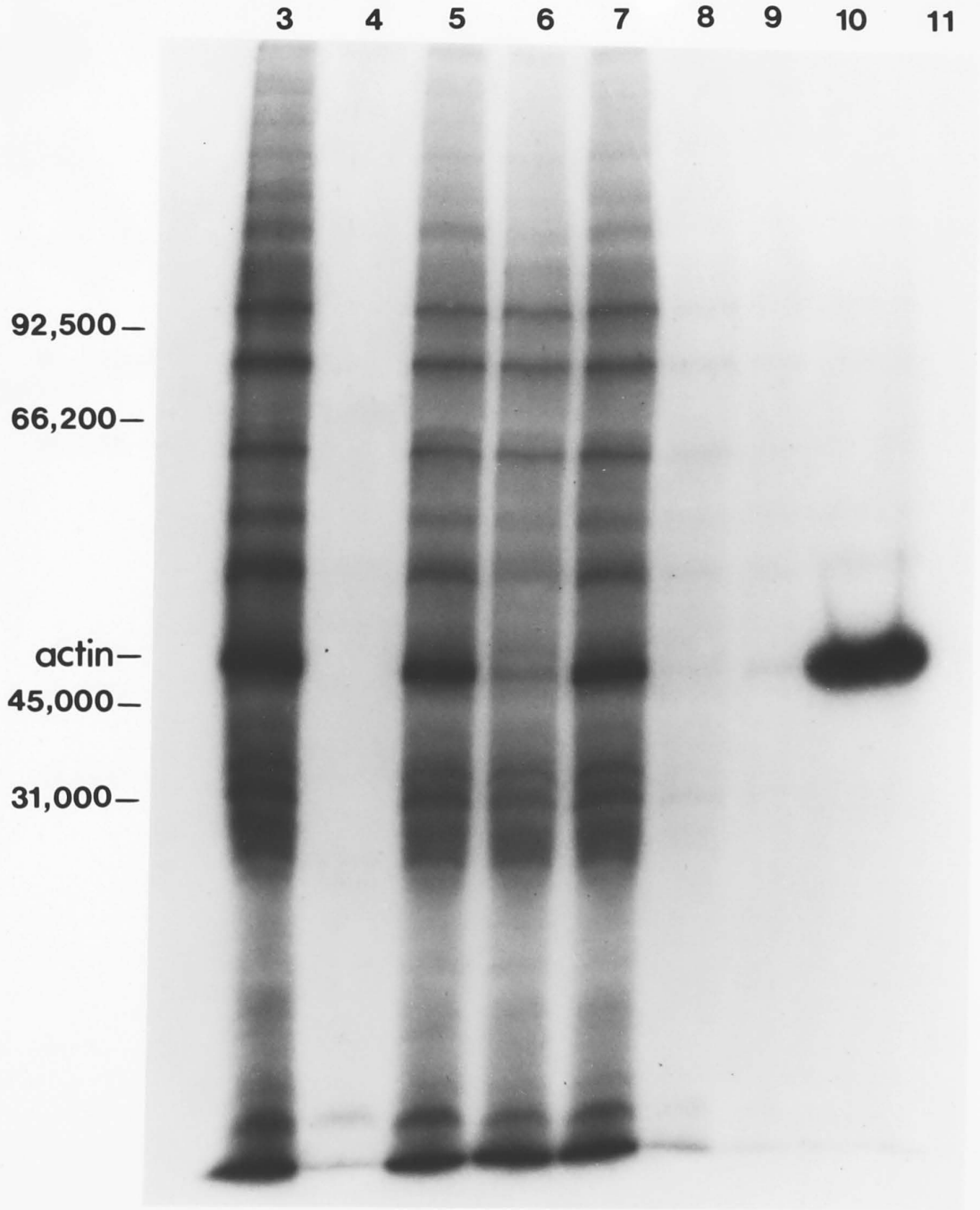
out in the presence of a range of Gu.HCl concentrations up to 3M. Maximal binding of actin occurred between 0.1 and 0.3M Gu.HCl (Fig. 12). The concentration of Gu.HCl chosen for the adsorption step was 0.18M.

The selective and quantitative removal of actin by binding to DNase I-Sepharose was confirmed by SDS-PAGE analysis (Fig. 13). The cell lysis/actin monomerization procedure effectively solubilized the actin as less than 1% of the total cellular actin was detectable in the nuclear pellet (Fig. 13b, lane 4) and less than 1% of the total radioactivity remained associated with the glass coverslip as insoluble material. Following adsorption to DNase I-Sepharose, 95% of the actin was removed from the supernatant (Fig. 13a, lane 6). The actin was not removed by adsorption to control uncoupled Sepharose (lane 7). The adsorbed actin was the only major protein bound to the DNase I-Sepharose (Fig. 13a, lane 10). Autoradiography for 72h demonstrated minor bands, some of which were also present in the control experiments (visible in the original autoradiogram but not in the photograph shown in Fig. 13b). However, levels of actin bound to the control Sepharose were estimated to be less than 1% of actin bound to DNase I-Sepharose and were therefore considered to be insignificant.

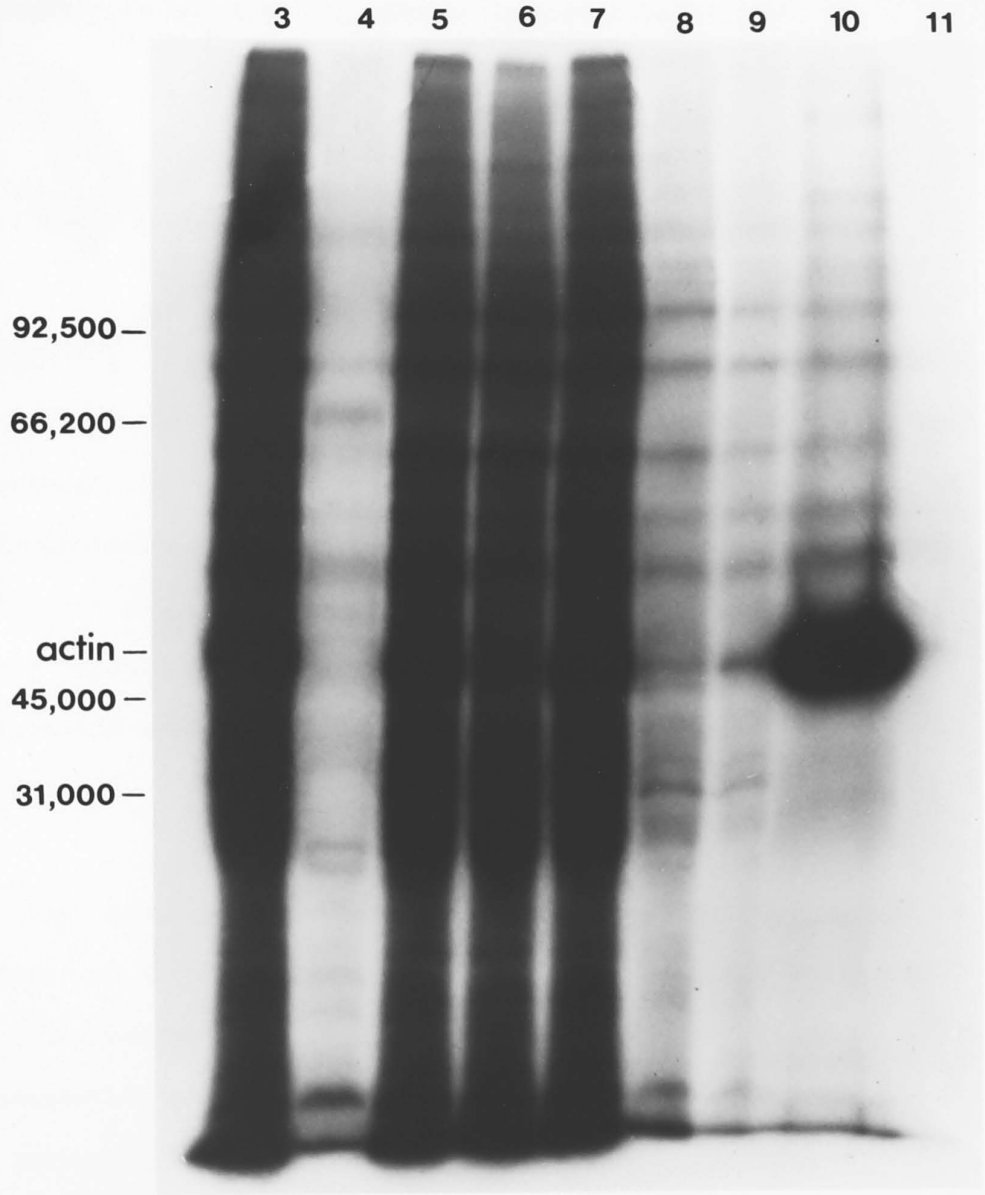
These experiments show that the presence of 0.18M GuHCl has no effect on the binding of actin to DNase I-Sepharose, that actin

FIGURE 13: Specificity of actin removal by adsorption to DNase I-Sepharose as analysed by SDS-PAGE and autoradiography.

The positions of molecular weight and actin standards are indicated. Cells were labelled with 70uCi of [^{35}S]methionine, lysed and the actin converted to actin monomer as described in section 3.2.2. The cell lysate (lane 3) was centrifuged for 10s in a microfuge to pellet nuclear material (lane 4). The supernatant (lane 5) after removal of the nuclear material was incubated with DNase I-Sepharose or control Sepharose. The material not adsorbed to the DNase I-Sepahrose is shown in lane 6 and that not adsorbed to the control Sepharose in lane 7. The Sepharoses were washed extensively with a high ionic strength wash buffer. Lanes 8&9 represent the material eluted from the DNase I-Sepharose and the control Sepharose, respectively, by the high ionic strength buffer. The material remaining adsorbed to the DNase I-Sepharose and control Sepharose was eluted with 1% (w/v) SDS at 100^{o}C (lanes 10&11, respectively). Autoradiography was carried out in the presence of AMPLIFY for (a) 24 and (b) 72 hours.



a



b

is specifically and quantitatively removed by adsorption to DNase I-Sepharose under these conditions, and that any persistent non-specific binding can be compensated for by subtraction of the radioactivity bound to the control uncoupled Sepharose.

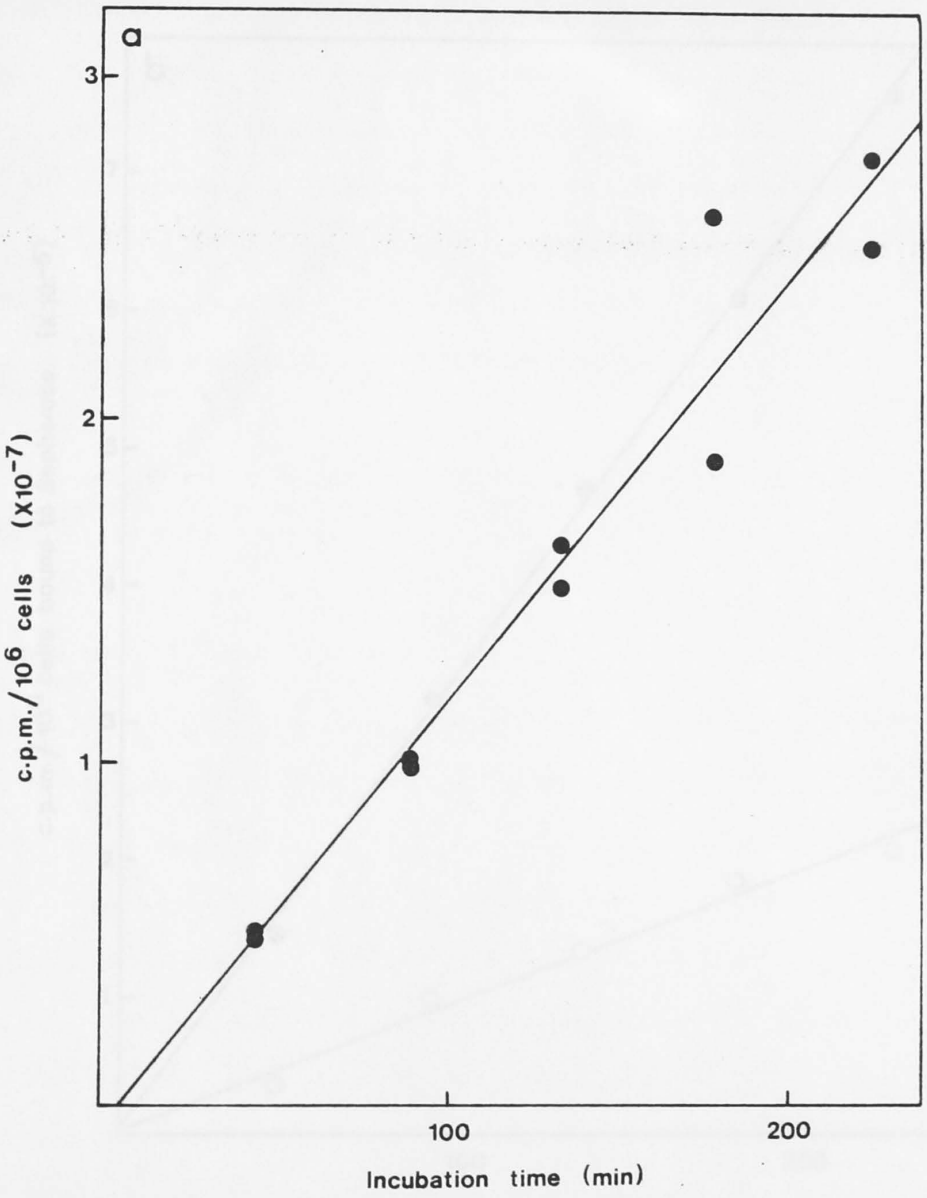
3.3.2 Actin Synthesis in Tumorigenic and Nontumorigenic Cells

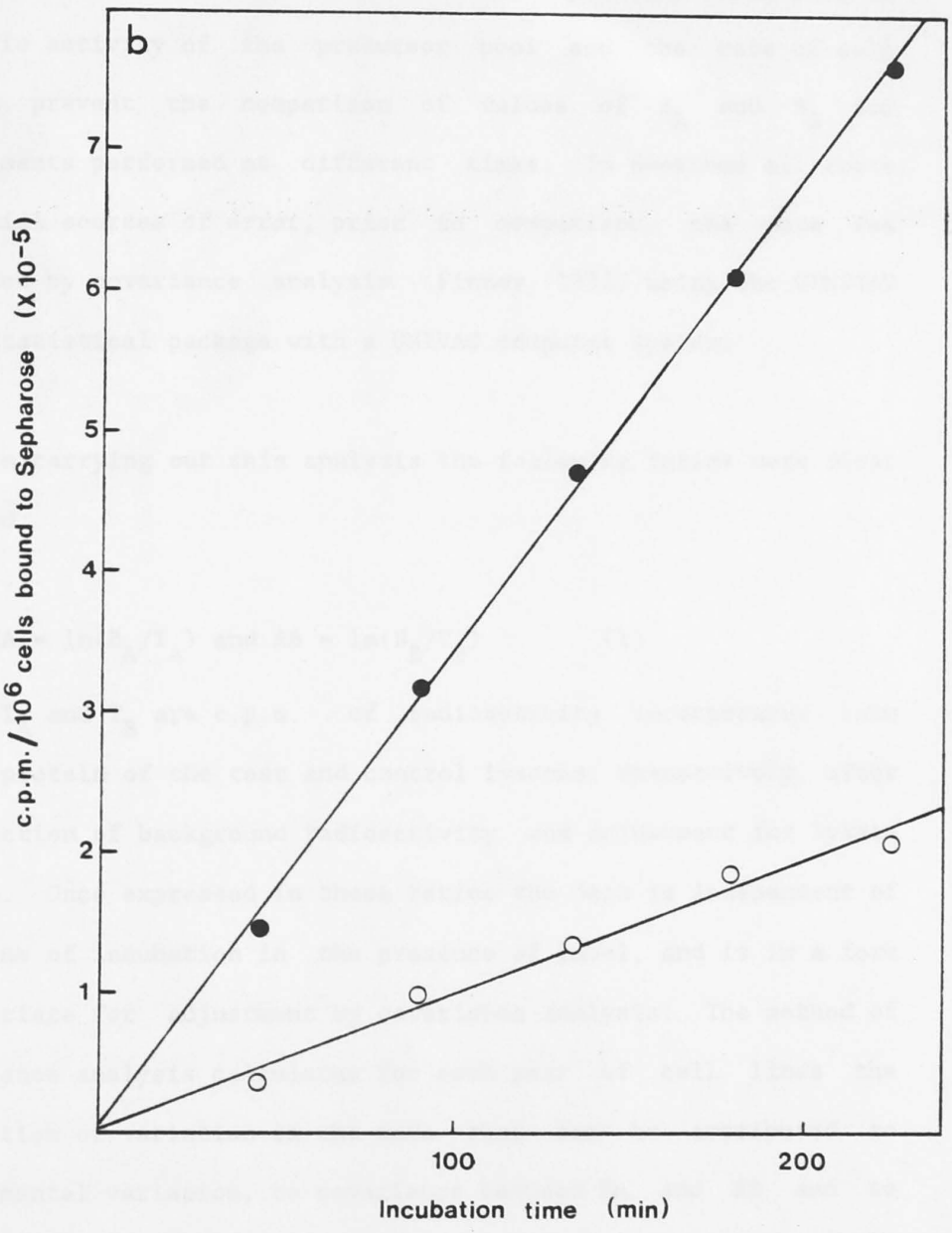
A representative set of results for one cell line from one experiment is shown in figure 14. The rate of incorporation of [^{35}S]methionine into both total protein and actin was always linear for at least 225min. Figure 14b shows the radioactive counts bound to DNase I-Sepharose and control Sepharose. Experiments were always repeated at least twice for each cell pair.

The radioactivity bound to DNase I-Sepharose (B_A) is a combination of the specific binding of actin to the DNase I ligand and the non-specific binding to the Sepharose support. The radioactivity bound to the Sepharose control (B_B) is a measure of the non-specific component only. However, B_A and B_B are not directly comparable as they are derived from independent coverslips. Consequently, the direct subtraction of B_B from B_A could introduce errors resulting from variations in the number of

FIGURE 14: Time courses of [^{35}S]methionine incorporation into total protein and actin.

The cells (5E) were incubated in the presence of 70uCi of [^{35}S]methionine and 3uM unlabelled methionine as described in section 3.2.2. [^{35}S]methionine incorporation into total cellular protein (a) and actin (b); (●) radioactivity bound to DNase I-Sepharose (○) radioactivity bound to control Sepharose.


a
3
2
1
c.p.m./10^6 cells (X10^-7)
100
200
Incubation time (min)


b
c.p.m. / 10^6 cells bound to Sepharose (X 10^-5)
1
2
3
4
5
6
7
100
200
Incubation time (min)

cells on each coverslip. In addition, day to day variations in specific activity of the precursor pool and the rate of cell growth prevent the comparison of values of B_A and B_B for experiments performed at different times. To overcome all these potential sources of error, prior to comparison, the data was adjusted by covariance analysis (Finney, 1952) using the GENSTAT 4.03 statistical package with a UNIVAC computer system.

In carrying out this analysis the following ratios were first defined:

$$RA = \ln(B_A/T_A) \text{ and } RB = \ln(B_B/T_B) \qquad (1)$$

where T_A and T_B are c.p.m. of radioactivity incorporated into total protein of the test and control lysates, respectively, after subtraction of background radioactivity and adjustment for lysate volume. Once expressed in these ratios the data is independent of the time of incubation in the presence of label, and is in a form appropriate for adjustment by covariance analysis. The method of covariance analysis calculates for each pair of cell lines the proportion of variation in the data that can be attributed to experimental variation, to covariance between RA and RB and to differences between the tumorigenic and nontumorigenic cells (Finney, 1952). This allows the calculation of further statistics: (1) RA_{ad}, the mean value of RA after adjustment for covariance with RB, (2) a combined standard error of the mean for the two RA_{ad} values calculated for each set of data, and (3) a t-statistic

(with 7 degrees of freedom for 10 data points) which can be used to test for a significant difference between the RA_{ad} values of the tumorigenic and nontumorigenic cells.

Adjustment of the data for the proportion of variation that is due to covariance of RB and RA effectively results in the subtraction of RB from RA. That is, without introducing errors due to direct subtraction, the contribution of non-specific binding to total binding is allowed for. The statistic RA_{ad}, is thus a measure of the proportion of total protein synthesis that is actin synthesis and will now be referred to as 'relative actin synthesis'.

A summary of the results of covariance analyses applied to two sets of experimental data for each of the cell pairs is shown in table 15. In three out of the 10 assays there is a significant difference between the relative actin synthesis in the nontumorigenic cells and the corresponding tumorigenic segregant with $P<0.05$ (marked by ** in Table 15). In one of the remaining 7 assays there is a significant difference, with $0.05<P<0.1$ (marked * in Table 15). However, there is no consistent difference between the relative actin synthesis of the tumorigenic and nontumorigenic cells.

The values of relative actin synthesis are expressed as natural logarithms. This results in a set of negative numbers

TABLE 15: A comparison of the relative actin synthesis values for the pairs of tumorigenic and nontumorigenic somatic cell hybrids.

Cell pair	Relative actin synthesis		Standard error of mean	t-statistic (7 d.f.)
	Tumorigenic	Nontumorigenic		
D98 AH2 + MRC-5	-3.144	-3.176	0.026	0.731
	-3.453	-3.388	0.035	0.738
5L + 5E	-3.507	-3.651	0.049	2.034*
	-3.340	-3.525	0.029	3.538**
ESH 39 + 39E C13	-3.440	-3.392	0.026	1.250
	-3.542	-3.503	0.035	0.475
IA3 cn TG + IA3 cn 2.1	-3.508	-3.341	0.043	0.922
	-3.398	-3.573	0.039	2.458**
5A7 mp 26.15 + CN2 B1 Col1	-4.113	-3.440	0.044	6.837**
	-3.651	-3.792	0.112	0.715

* Denotes a significant difference with $0.05<P<0.1$;

** Denotes a significant difference with $P<0.05$.

from which it is difficult to extract any meaning. Taking the antilog of relative actin synthesis produces a statistic, termed the 'mean actin synthesis' which is the proportion of total protein synthesized that is actin (Table 16). The total actin content of the tumorigenic cells is, in all instances, less than that of the corresponding nontumorigenic cells. From the total actin content of the tumorigenic and nontumorigenic cells along with the mean actin synthesis of the nontumorigenic cells, it is possible to calculate the mean actin synthesis that would be expected for the tumorigenic cells should the reduced total actin content be due only to a suppression of actin synthesis.

That is,

$$\text{expected}_T = [\text{actin}]_T/[\text{actin}]_{NT} \text{ X } \text{observed}_{NT} \qquad (2)$$

where expected_T is the mean actin synthesis expected for the tumorigenic cells; $[\text{actin}]_T$ and $[\text{actin}]_{NT}$ are the total actin content of the tumorigenic and nontumorigenic cells, respectively, and observed_{NT} is the value of mean actin synthesis determined for the nontumorigenic cells.

A comparison of the observed and expected values of mean actin synthesis is shown in table 16. In nine out of 10 assays, the observed mean actin synthesis did not reach the value expected, should the difference in total actin content be due to a

TABLE 16: A comparison of the observed and predicted mean actin synthesis of the tumorigenic and nontumorigenic somatic cell hybrids.

Cell line	Status	Total actin* content (pg/cell)	Observed actin synthesis	Predicted actin synthesis
MRC-5	NT	17.3	0.042	-
			0.034	-
D98 AH2	T	9.0	0.043	0.022
			0.032	0.018
5E	NT	16.0	0.026	-
			0.030	-
5L	T	9.7	0.030	0.016
			0.035	0.018
39E C13	NT	15.0	0.034	-
			0.030	-
ESH 39	T	10.8	0.032	0.024
			0.029	0.022
IA3 cn 2.1	NT	15.1	0.035	-
			0.028	-
IA3 cn TG	T	9.8	0.030	0.023
			0.033	0.018
CN2 B1 Coll	NT	11.5	0.032	-
			0.023	-
5A7 mp 26.15	T	8.2	0.016	0.023
			0.026	0.016

NT denotes nontumorigenic and T denotes tumorigenic, as assessed by the injection of $5x10^6$ cells subcutaneously into nude mice.

* For a detailed description of the determination of actin content, see chapter 2.

suppression of actin synthesis alone.

Since the nontumorigenic and tumorigenic hybrid cells were derived from the same malignant parental cell lines, the mechanism of tumorigenic transformation might be expected to be the same or similar in all cases. For this reason the values of relative actin synthesis for the cells were grouped and compared according to their tumorigenic phenotype (Table 17). The average relative actin synthesis for the tumorigenic cells was not significantly different from the value for the nontumorigenic cells. This suggests that the reduced actin content of the tumorigenic cell lines is *not* the result of reduced actin synthesis.

3.4 Discussion

The steady state concentration of any protein is the result of the balance between its rates of synthesis and degradation. Consequently, any alteration in the steady state level must be due to a change in one or both of these processes. The data presented in this chapter show that actin synthesis, relative to total protein synthesis, is not decreased in the tumorigenic cells compared with the nontumorigenic cells. Indeed, in the particular

TABLE 17: A comparison of the relative actin synthesis values according to tumorigenicity.

	Relative actin synthesis	
	Tumorigenic	Nontumorigenic
	-3.144	-3.176
	-3.453	-3.388
	-3.507	-3.651
	-3.340	-3.525
	-3.440	-3.392
	-3.542	-3.503
	-3.508	-3.341
	-3.398	-3.573
	-4.113	-3.440
	-3.651	-3.792
Totals	-35.096	-34.781
n	10	10
Means	-3.510	-3.478

Pooled variance of means, $s_{x1-x2}=0.0965$

t-statistics = $(X_1-X_2)/s_{x1-x2}=0.3264$

case of the 5E/5L pair, the tumorigenic 5L cell line shows a significantly greater synthesis of actin. Therefore, the reduced total actin content of the tumorigenic cells is not the result of the specific suppression of actin synthesis.

It could be argued that the assay methods used in these, and the experiments described in Chapter 2, do not detect a fraction of the actin in the tumorigenic cells. That is, a portion of the total cellular actin of the tumorigenic cells is not converted to monomer and is thus unable to bind to DNase I. However, SDS-PAGE analysis shows that less than 10% of the total cellular actin does not become adsorbed to the DNase I-Sepharose, and this figure includes estimates of actin lost as a result of binding to the nuclei or membrane vesicles as well as actin not adsorbed from the cell lysate supernatant. Hence the presence of a pool of actin not detectable by binding to DNase I and large enough to explain the 35% reduction in total actin content seems unlikely.

It must be concluded, therefore, that the rate of actin degradation in the tumorigenic cells is greater than that in the nontumorigenic cells. The rate of degradation of actin is slow in both muscle and non-muscle cells with the $t_{1/2}$ of actin in cardiac muscle cultures being 4.7 days (Clark & Zak, 1981) and in growing 3T3 and SV40-transformed 3T3 cells more than 3 days (Fine & Taylor, 1976). However, the direct measurement of the actin degradation rate in the somatic cell hybrids was hampered by the

slow reduction in the specific activity of [^{35}S]methionine-labelled actin, compared with the fast cell-doubling time (about 24h) exhibited by the HeLa/fibroblast somatic cell hybrids. The reduction in the specific activity of actin due to cell division prevented the application of a simple procedure, such as that used for assessing actin synthesis, to obtain a measure of actin degradation in the tumorigenic and nontumorigenic cells.

It is of interest to consider the mechanism(s) by which the proposed increased rate of actin degradation could be brought about. The presence in the nontumorigenic cells of highly organized, stable actin structures such as stress fibres should result in a slow rate of turnover of actin polymer with actin monomer. The reduced microfilament organization associated with tumorigenicity may cause an increased rate of turnover of actin polymer with monomer. Since actin monomer is known to be much less stable and more susceptible to proteolysis than actin polymer (Lehrer & Kerwar, 1972; Rich & Estes, 1976) it is possible that an increased rate of turnover of actin monomer with actin polymer results in an increased availability of actin monomer for degradation thereby leading to a decreased total actin content in the tumorigenic cells.

While the data discussed in this chapter indicate that the synthesis of actin is not suppressed in the tumorigenic cells, it

remains possible that the pattern of actin isoforms is altered in the tumorigenic cells. If the different actin isoforms have different functions and intracellular distributions, a change in the ratio of isoforms could have a dramatic effect. Increased production of an isoform that tends to incorporate into dynamic structures, rather than stable structures such as stress fibres, could result in the instability of actin structures, leading to the increased turnover of actin polymer with monomer and therefore an increased availability of actin monomer for degradation. However, until such differences in function or distribution of actin isoforms have been demonstrated, such mechanisms must remain highly speculative.

Kakunaga *et al.* (1984) have described a mutated beta-actin, isolated from a chemically transformed human fibroblast cell line. This mutated actin possesses one additional negative net charge and has a diminished ability to incorporate into the detergent-resistant cytoskeleton but while its rate of synthesis is increased the mutant actin has a much shorter half-life in the cell (Leavitt *et al.*, 1982) suggesting a rapid rate of degradation. This may be the result of the inability of this abnormal actin to become incorporated into the cytoskeleton thereby increasing its susceptibility to proteolysis. However, the actin within the tumorigenic Hela/fibroblast hybrid cells is apparently functionally competent since reverse transformation with dexamethasone or sodium butyrate induces the formation of

stable stress fibres (Der *et al.*, 1981).

The organization of actin is controlled at all levels of organization from monomer to complex multifilamentous structures, by a range of actin-binding proteins. Changes in the activity of any of these regulatory proteins could lead to an instability of F-actin structure, resulting in increased turnover of actin polymer with monomer and subsequently increased actin degradation. The proteins vinculin, alpha-actinin and talin are implicated in the attachment of stress fibres to the plasma membrane. Disruption of this attachment mechanism may prevent the formation of stable stress fibres. Gelsolin, fragmin and related proteins, through capping, severing and nucleating activities control actin filament length. These proteins could potentially cause the breakdown of F-actin structures. Tropomyosin, by binding to F-actin, stabilizes and protects F-actin structures against the severing actions of proteins such as gelsolin. Reduced levels of tropomyosin would therefore leave stress fibres susceptible to the severing proteins. The formation of F-actin structures such as stress fibres and three-dimensional networks requires the presence of crosslinking and bundling proteins. Deficiencies in these proteins would prevent the formation of such structures.

3.5 Conclusions

The reduced total actin content of tumorigenic cells, relative to nontumorigenic cells, is not the result of the specific suppression of actin synthesis. While it was not practicable to measure the rate of actin degradation, it is concluded that the reduced actin content in tumorigenic cells is most likely to be the result of an increased rate of actin degradation.

The rate of protein degradation could be increased as a result of an increased turnover of actin polymer with actin monomer resulting from the instability of microfilament structure in the tumorigenic cells. That is, the reduced actin content of the tumorigenic HeLa/fibroblast hybrid cells is a consequence and not the cause of the disorganized microfilament system seen in these cells. Therefore the emphasis of further work returns to the cause of the microfilament disorganization in the tumorigenic cells.

While alterations in the balance of actin isoforms or mutations of the actin molecule itself may influence microfilament

structure and function, it is more likely that the various actin-binding proteins are the major factor in determining microfilament organization. Indeed, any differences in activity of the actin isoforms, or mutated actins, may be through differing interactions with actin-binding proteins. The various actin isoforms are highly homologous proteins with small differences only in the amino acid sequence of a region near the amino terminal. If this region of the actin molecule is important for interaction with actin-binding proteins, then any effect of altered isoform ratios or the synthesis of a mutated actin molecule on microfilament organization could be through disruption of the normal activities of the actin-binding proteins.

Chapter 4

ACTIN-BINDING PROTEINS IN TUMORIGENIC AND NONTUMORIGENIC HUMAN SOMATIC CELL HYBRIDS.

4.1 Introduction

The intracellular concentration of any protein at steady state results from the balance between the its synthesis and degradation. The reduced total actin content of the tumorigenic HeLa/fibroblast hybrid cells is not due to the specific suppression of actin synthesis (see chapter 3). This suggests that the rate of actin degradation must be increased in the tumorigenic cells.

Actin monomer is more susceptible to proteolysis and irreversible unfolding than is actin polymer (Rich & Estes, 1976; Lehrer & Kerwar, 1972) and actin polymer is in equilibrium with actin monomer. Consequently, destabilization of the multifilamentous structures, such as stress fibres, *in vivo* may, through an increase in the number of filament ends, result in an increased rate of turnover of actin polymer with actin monomer, thereby increasing the availability of actin monomer for degradation. Therefore, it is most likely that the presumed increase in actin degradation and subsequent reduction in total actin content that was observed in the tumorigenic somatic cell hybrids is a consequence of the loss of stable microfilament

structures in the tumorigenic cells.

The organization of actin is regulated at all levels by a diversity of actin-binding proteins (Pollard & Cooper, 1986). The combination of the different activities exhibited by these actin-binding proteins regulates actin organization with such a degree of sensitivity that relatively minor changes in net activity of the actin-binding proteins can induce major changes in actin organization. For example, the formation of high viscosity gels from actin filaments occurs at a critical concentration of crosslinking protein, relative to the length of actin filaments being crosslinked. Consequently, abrupt transitions between sol and gel states are induced by variations in mean actin filament length (Yin & Stossel, 1982). Shortening of actin filaments can occur through the calcium-sensitive capping and severing activity of gelsolin and related proteins, or through the activity of the low molecular weight depolymerizing proteins. Inhibition of these activities allows reannealing of the short filaments to occur with a subsequent return to the gel state.

Treatment of the tumorigenic HeLa/fibroblast hybrid cells with dexamethasone or sodium butyrate induces the organization of the microfilaments into stress fibres (Der *et al.*, 1981). This indicates that the actin in the tumorigenic cells is, under certain conditions, able to form stable polymeric structures. Therefore, the absence of these stable polymeric structures in

tumorigenic cells under normal tissue culture conditions is unlikely to be due to an abnormality in actin structure, function or relative isoform expression. Rather, the disorganized microfilament system seen in the tumorigenic cells is most likely to be the result of differences in the actin-binding proteins, differences which can be reversed or overcome by the effects of dexamethasone or sodium butyrate.

Changes in actin-binding proteins have been previously reported to be associated with malignant transformation. A reduction in tropomyosin content has been observed in several transformed cell lines, with the higher molecular weight isoforms tending to be lost (Lin *et al.*, 1985). Tropomyosin stabilizes F-actin structures and protects them against the severing action of proteins such as gelsolin. Consequently, the loss of tropomyosin could be a factor in the disorganization of the microfilament system that is typical of transformed cells. Crude cell lysates from tumorigenic HeLa/fibroblast cell lines were found to exhibit significantly more actin nucleating activity than extracts from the nontumorigenic cell lines. In addition, the final extent of actin polymerization, relative to pure actin controls, was reduced to a greater extent in the presence of cell extracts from the tumorigenic cells (Tellam & Banyard, 1986). This suggests that the tumorigenic HeLa/fibroblast hybrid cells contain greater amounts of a barbed-end actin filament capping protein, such as gelsolin, compared with the nontumorigenic cells.

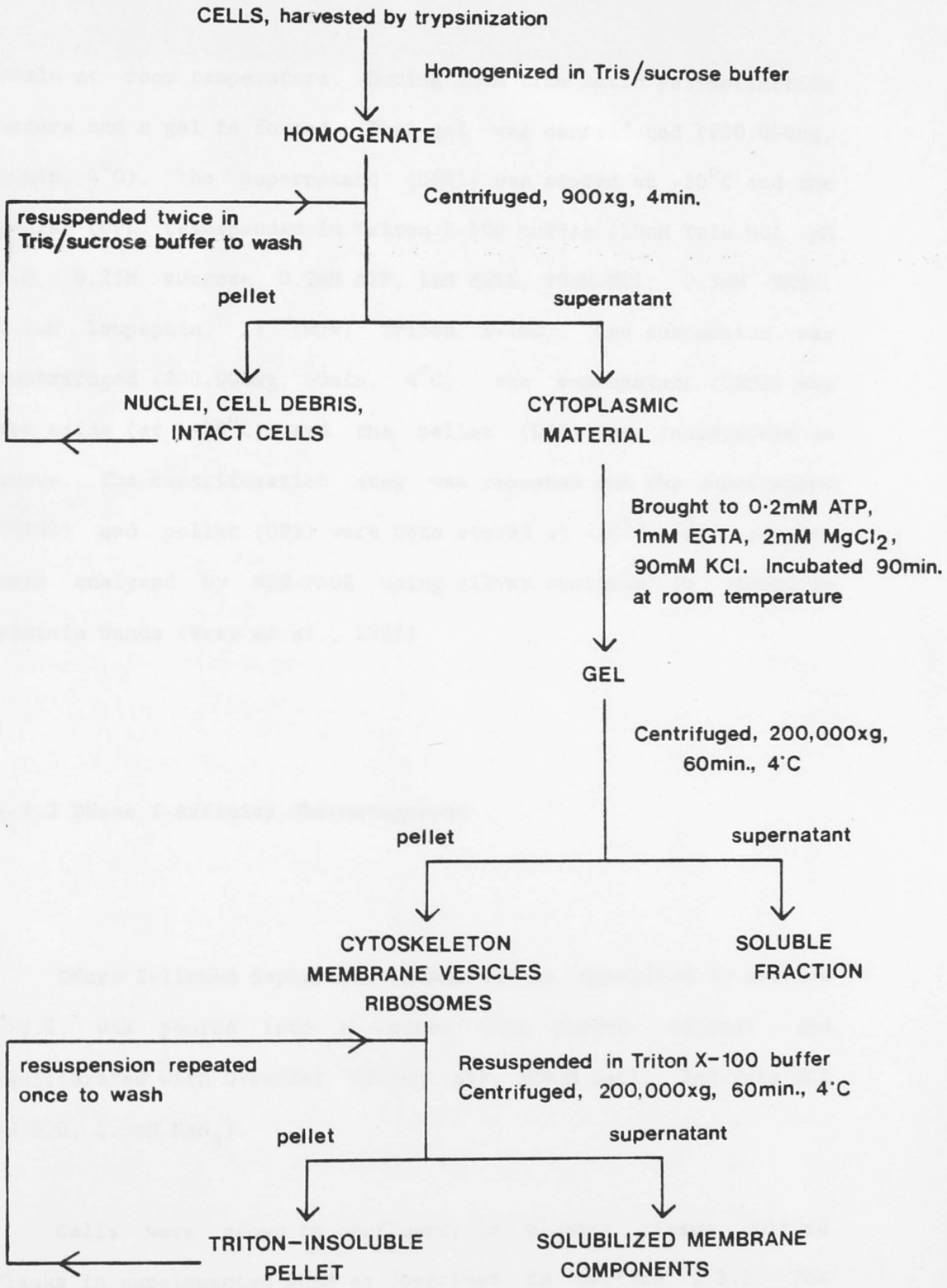
To compare the actin-binding proteins of the tumorigenic and nontumorigenic HeLa/fibroblast hybrid cells, several procedures were used to enrich for different pools of actin-binding proteins. Conventional methods for the preparation of the Triton-insoluble cytoskeleton were adapted to produce a Triton-insoluble pellet enriched for actin-binding proteins, in particular those actin-binding proteins associated with membranes. A pool of actin monomer-binding proteins was selected by DNase I-affinity chromatography while high speed centrifugation was used to produce a pellet enriched for F-actin and F-actin-binding proteins. A comparison was made of the protein composition of these pools from tumorigenic and nontumorigenic HeLa/fibroblast cells but, in addition, assays of actin-binding protein activity were used with a view to the identification of differences in activity that might cause the altered microfilament organization seen in the tumorigenic cells. The emphasis of these investigations is placed on actin capping, severing and depolymerizing activities since such activities might be expected to disrupt polymeric actin structures and increased levels of capping activity have been previously detected in the tumorigenic HeLa/fibroblast hybrid cells (Tellam & Banyard, 1986).

4.2 Materials and Methods

4.2.1 Preparation of the Triton-Insoluble Pellet

Cells were grown to confluency in plastic tissue culture flasks in supplemented DMEM as described in section 2.2.1. The adherent cells were detached from the tissue culture flasks with trypsin and EDTA, washed twice in PBS and counted as described in section 2.2.4. The final cell pellet was resuspended in Tris/sucrose buffer (10mM Tris.HCl pH 8.0, 0.25M sucrose, 0.5mM PMSF, 0.1uM leupeptin) at a density of $1x10^7$ cells/mL. The cells were lysed (Fig. 15) by homogenization (20 strokes at 3000 r.p.m. using a size AA glass grinding vessel from Arthur H. Thomas Company, Philadelphia, U.S.A.) and nuclei, intact cells and cellular debris were pelleted by centrifugation (900xg, 4min). The pellet was resuspended in Tris/sucrose buffer, homogenized again (5 strokes at 3000 r.p.m. using a size AA grinding vessel), centrifuged (900xg, 4min) and the supernatants were then combined. This procedure was repeated once more with the pellet being resuspended by pipetting, not homogenization. The final nuclear pellet was discarded and the supernatant (NSN) was brought to 0.2mM ATP, 1mM EGTA, 2mM $MgCl_2$ and 90mM KCl then incubated for

FIGURE 15: An outline of the method used to prepare the Triton-insoluble pellet.


CELLS, harvested by trypsinization
Homogenized in Tris/sucrose buffer
HOMOGENATE
Centrifuged, 900xg, 4min.
resuspended twice in Tris/sucrose buffer to wash
pellet
supernatant
NUCLEI, CELL DEBRIS, INTACT CELLS
CYTOPLASMIC MATERIAL
Brought to 0·2mM ATP, 1mM EGTA, 2mM $MgCl_2$, 90mM KCl. Incubated 90min. at room temperature
GEL
Centrifuged, 200,000xg, 60min., 4°C
pellet
supernatant
CYTOSKELETON MEMBRANE VESICLES RIBOSOMES
SOLUBLE FRACTION
resuspension repeated once to wash
Resuspended in Triton X-100 buffer
Centrifuged, 200,000xg, 60min., 4°C
pellet
supernatant
TRITON-INSOLUBLE PELLET
SOLUBILIZED MEMBRANE COMPONENTS

90min at room temperature. During this time actin polymerization occurs and a gel is formed. This gel was centrifuged (200,000xg, 60min, 4°C), the supernatant (USN1) was stored at -20°C and the pellet (UP1) resuspended in Triton X-100 buffer (10mM Tris.HCl pH 8.0, 0.25M sucrose, 0.2mM ATP, 1mM EGTA, 90mM KCl, 0.5mM PMSF, 0.1uM leupeptin, 1% (w/v) Triton X-100). The suspension was centrifuged (200,000xg, 60min, 4°C), the supernatant (USN2) was set aside (at -20°C) and the pellet (UP2) was resuspended as above. The centrifugation step was repeated and the supernatant (USN3) and pellet (UP3) were both stored at -20°C. All samples were analysed by SDS-PAGE using silver staining to visualize protein bands (Wray *et al.*, 1981).

4.2.2 DNase I-Affinity Chromatography

DNase I-linked Sepharose, prepared as described in section 3.2.1, was poured into a column (7mL packed volume) and equilibrated with G-buffer (200uM ATP, 200uM $CaCl_2$, 2mM Tris.HCl pH 8.0, 1.5mM NaN_3).

Cells were grown to confluency in plastic tissue culture flasks in supplemented DMEM as described in section 2.2.1. The cells were harvested with trypsin and EDTA, washed twice in PBS

and counted as described in section 2.2.4. The final cell pellet was resuspended in ice-cold sonication buffer (G-buffer plus 1mM DTT and 10uM leupeptin) containing 0.125mM PMSF. All subsequent procedures were carried out at 4°C.

The cells were lysed by sonication (3x20s with 10s pauses with output control set at mark 6 on a Sonicator Model W-220F from Heat Systems, Ultrasonics, Inc., Plainview, New York, U.S.A.) and left for 10min. The nuclei and cell debris were pelleted by centrifugation (10min in a microfuge). The lysate supernatant was applied to the DNase I-Sepharose column and the column subsequently washed with sonication buffer. A flow rate of 0.5mL/min was maintained until the major peak of unbound protein (the flow-through) had been eluted from the column, as determined by monitoring the A_{280} of the column eluate. The flow-rate was then increased to 1mL/min. The column was washed with a total of 70mL of sonication buffer, then proteins bound with low affinity were eluted with 70mL of 0.1M NaCl buffer (2mM $CaCl_2$, 2mM Tris.HCl pH 7.8, 5mM NaN_3, 0.1M NaCl, 1mM DTT and 10uM leupeptin). Proteins bound with higher affinity were eluted with 70mL of 0.6M NaCl buffer (2mM $CaCl_2$, 2mM Tris.HCl pH 7.8, 5mM NaN_3, 0.6M NaCl, 1mM DTT and 10uM leupeptin) and actin and associated proteins were eluted with Gu.HCl buffer (10mM Tris.HCl pH 7.6, 5mM $CaCl_2$, 3M Gu.HCl). A total of 40mL of Gu.HCl buffer was passed through the column to ensure all protein bound to the DNase I was removed. The column was then re-equilibrated with sonication buffer.

The column eluate was collected (5mL fractions) and 100uL aliquots of each fraction were assayed for protein content by the Micro-Bradford method. Fractions containing protein were pooled and dialysed overnight against 0.05M NH_4HCO_3, lyophilized, and finally stored at -20°C.

4.2.3 The Micro-Bradford Method of Protein Determination

A stock solution of 10ug/mL bovine serum albumin (BSA) in distilled water was prepared and the precise concentration determined using an extinction coefficient of 5.8 absorbance unit at 280nm for a 1% solution. Using this stock solution, standards containing between 0 and 0.7ug BSA in 100uL were prepared in a flat-bottomed 96-well multi-tray. Samples were diluted to a final volume of 100uL with distilled water then 100uL of Coomassie reagent (0.06% (w/v) Coomassie Blue G-250 in 1.9% (w/v) perchloric acid) was added and the solutions were mixed by placing the tray on a shaker for 30s. After 2min the absorbance ratio (630/405nm) was read on a Microelisa Auto Plate Reader. Colour development was complete at 2min and stable for 30min (Rylatt & Parish, 1982).

4.2.4 Falling-Ball Viscometry

Lyophilized samples (cell lysates or actin-binding protein preparations), solubilized in 250mM NaCl buffer (250mM NaCl, 10mM Tris.HCl pH 8.0) were assayed by falling-ball (low shear) viscometry in the presence of 200 ug/mL (5uM) actin, 0.5mM ATP, 0.5mM $CaCl_2$, 5mM $MgCl_2$, 125mM NaCl and 6mM Tris.HCl, pH 8.0. Assays were prepared by mixing 200uL of 250mM NaCl buffer (containing the sample), 20uL of 100mM $MgCl_2$, about 100ug of G-actin in G-buffer, and G-buffer to a final volume of 400uL. The G-actin (prepared as described in section 2.2.3) was added last to initiate polymerization and the solution was drawn up into capillary tubes (1.5mm diameter by 15cm long) held in a vertical position. After 30 or 60min at room temperature the low shear viscosity of the actin solution was measured as the time taken for a ball-bearing (0.6mm diameter from The New England Miniature Ball Co., Norfolk, C.T., U.S.A.) to fall 12cm down the capillary tube. Since this process causes shearing of the actin filaments, multiple measurements were spaced by at least 30min to allow the filaments to reanneal.

4.2.5 Assay of Actin Polymerization by Pyrene-Actin Fluorescence Enhancement

Lyophilized samples (cell lysates or actin-binding protein preparations) were taken up in 100uL G-buffer and insoluble material was pelleted by centrifugation (1min in a microfuge). The total protein concentration of the supernatant was determined by the Micro-Bradford method using a 5uL sample. N-(1-pyrenyl)iodoacetamide-labelled actin (pyrene-actin) was prepared according to the method described by Tellam and Frieden (1982) and stored lyophilized. Solutions of monomeric pyrene-actin and unlabelled rabbit skeletal muscle actin were freshly prepared from lyophilized powder as described in section 2.2.3.

Trace quantities of pyrene-actin (typically 10ug/mL or 0.24uM) were used in conjunction with unlabelled actin (10uM) to measure the large fluorescence enhancement (excitation at 365nm and emission at 407nm) associated with the incorporation of pyrene-actin into actin polymer. This fluorescence enhancement is a direct measure of the kinetics of actin polymerization (Tellam & Frieden, 1982). This method was used to assess the effect of samples containing actin-binding proteins (4ug/mL total protein)

on the time-course of actin polymerization. The assays were carried out using a Perkin-Elmer model LS-5 Luminescence spectrophotometer.

4.2.6 Western Blotting

Proteins which were separated by SDS-PAGE were transferred to nitrocellulose (35mA, 25V) overnight in transfer buffer (9.6mM Tris pH 8.4, 78mM glycine, 20% (v/v) methanol). Two sheets of nitrocellulose were used with the primary sheet (that closest to the acrylamide gel) being used for blotting with antibodies. Proteins present in large amounts will saturate the binding capacity of the primary sheet but will be adsorbed onto the secondary (backing) sheet. This secondary sheet of nitrocellulose was washed for 5min with distilled water, stained for 30s in nitrocellulose Coomassie stain (0.2% (w/v) Coomassie Brilliant Blue R, 40% (v/v) methanol, 10% (v/v) acetic acid) and rinsed twice in destain (90% (v/v) methanol, 2% (v/v) acetic acid) for 10min. The staining of proteins adsorbed to the secondary sheet of nitrocellulose indicates whether the transfer procedure has been successful. The gel was stained with Coomassie Blue (0.05% (w/v) Coomassie Brilliant Blue R, 47.6% (v/v) methanol, 4.76% (v/v) acetic acid) and destained (9% (v/v) acetic acid, 45.5% (v/v)

methanol). If proteins have been successfully eluted out of the gel, minimal protein staining will be seen.

The primary sheet of nitrocellulose was rinsed for 5min in distilled water then blocked for 1h at 37°C with 20% (w/v) skim milk powder (Diploma brand) in Tris-buffered saline (TBS: 25mM Tris.HCl pH7.4, 140mM NaCl, 3mM NaN_3). The nitrocellulose was rinsed for 5min in TBS and then incubated for 1h at room temperature with primary antibody (affinity purified rabbit antigelsolin) in 20% (w/v) skim milk in TBS. After rinsing the sheet twice (5min each) with TBS containing 0.05% (v/v) Tween-20 and once (5min) in TBS, the nitrocellulose was incubated with 5uL of secondary antibody (Bio-Rad Goat anti-rabbit IgG (H+L), alkaline phosphatase conjugate, Western Blotting grade, affinity purified from Bio-Rad Laboratories, Richmond, Calif., U.S.A.) in 15mL 20%(w/v) skim milk in TBS for 1h at room temperature. The nitrocellulose was then rinsed twice (5min each) in TBS containing 0.05%(w/v) Tween-20, once (5min) in TBS and once (5min) in distilled water. All washes and incubations were carried out with continuous shaking.

The alkaline phosphatase reagent was prepared while completing these rinsing steps: 15mL of AP 9.5 buffer (0.1M Tris.HCl pH 9.5, 0.1M NaCl, 5mM $MgCl_2$) was warmed slightly (37°C) and 5mg NBT (Nitro Blue Tetrazolium Grade III from Sigma Chemical Co., St. Loius, Mo. U.S.A.) was dissolved in 1mL of the warmed

AP9.5 buffer. This suspension was vortexed lightly and the residue spun off (1min in a microfuge) and the supernatant was transferred to the warmed AP9.5 buffer. This step was repeated twice more. Between 2 and 5mg of BCIP (5-bromo-4-chloro-3-indolyl phosphate, p-toluidine salt from Sigma Chemical Co.) was dissolved in 50uL of dimethlyformamide and added dropwise to the NBT/AP9.5 solution. The nitrocellulose was incubated with this BCIP/NBT/AP9.5 buffer solution for 30-60min at room temperature, with mixing and protected from direct light, or until the reactive proteins are visible as brown bands. The reaction was stopped by rinsing the nitrocellulose 2-3 times in distilled water.

4.2.7 Dot Blot

Lyophilized samples of interest were taken up in G-buffer and spotted onto circles of nitrocellulose arranged on blotting paper. The nitrocellulose was allowed to dry before being transferred to 96-well multi-dishes and then treated with antibodies as described in section 4.2.6.

4.2.8 The Growth of Cells on Cytodex-3 Microcarriers

'Repelcote' water repellant (2% dimethyldichlorosilane in 1,1,1-trichloroethane from Hopkin and Williams, Essex, England), was used to siliconize all glassware. Cytodex-3 microcarriers were obtained from Pharmacia Fine Chemicals, AB, Uppsala, Sweden.

The dry microcarrier beads were swollen in PBS (100mL/g) for at least 3h at room temperature. The PBS was decanted and replaced with fresh PBS (50mL/g) and then the microcarriers were sterilized by autoclaving (115^{o}C, 15min, 15 p.s.i.). The PBS was decanted from 2g Cytodex-3 and 150mL culture medium (DMEM supplemented as described in section 2.2.4) was added and then warmed to 37^{o}C. The seeding cell suspension (about 1x10^{7} cells/2g Cytodex) was then added and the microcarrier/cell suspension (gassed with 5% CO_2 in air) was incubated for 1h at 37^{o}C with mixing (by rotary shaker). The microcarrier suspension was then transferred to a culture flask, the volume increased to 450mL with culture medium, and gassed with 5% CO_2 in air. The suspension was incubated at 37^{o}C with stirring by bar magnet contained in a bulb-shaped rod suspended in the flask (See 'Microcarrier Cell Culture, Principles and Methods' from Pharmacia Fine Chemicals, AB, Uppsala, Sweden for details of appropriate methods and culture

vessels).

Cell growth was monitored by microscopic examination after the addition of a drop of Trypan Blue to a drop of Cytodex bead suspension to enhance visualization of the cells. Cell growth was continued until the cells were judged to be confluent on the microcarriers, with fresh culture medium being added after three to four days.

Cells were harvested using the following procedure. The microcarriers were allowed to settle, the media was decanted and the microcarriers washed in 400mL PBS. After decanting the PBS, the microcarriers were resuspended in 200mL PBS containing 0.025% (w/v) trypsin and 0.02% (w/v) EDTA and incubated for 15min at 37^{o}C. The microcarriers were separated from the cells by filtration through nylon gauze and the cells were then pelleted by centrifugation (550xg, 4min). The cells were washed twice by resuspending in PBS and then pelleting by centrifugation. A sample of the cell suspension in PBS was taken for cell counts using the Trypan Blue exclusion method and a haemocytometer.

4.2.9 Reversed-Phase High Pressure Liquid Chromatography

All solvents were High Pressure Liquid Chromatography (HPLC) grade from J.T. Baker Chemical Co., Phillipsburg, N.J., U.S.A. and high purity water was prepared by passing quartz distilled water through a mixed-bed resin. Solvents used in HPLC analysis were filtered and degassed prior to use with a Millipore filtration unit, under a -70kPa vacuum generated by a Millipore pump using Millipore HVHP 004700 filters (organic, 0.45um pore size) for methanol and acetonitrile, and HAWP 04700 (aqueous, 0.45um pore size) for water. Trifluoroacetic acid (TFA) was added after filtration and degassing. All HPLC equipment was from Waters Associates, Massachusetts, U.S.A., consisting of: Waters 840 Data and Chromatography control station (incorporated into a Digital Electronics Corp. Professional 350 series computer), two 510 series HPLC pumps, WISP 710B auto sampler (200uL sample loop), and a 490 programmable multiwavelength detector and the Systems Interlink Module.

HPLC separations were performed using a Vydac Protein C4 reversed-phase column (214TP series, C4 bonded, 300-Angstrom pore size, 4.6mm I.D. x 25cm length) from the Sep/a/ra/tions Group, Hesperia, CA, U.S.A. (obtained through Edwards Instrument Company,

Narellan, N.S.W., Australia). A Frac-100 fraction collector (from Pharmacia Fine Chemicals AB, Uppsala, Sweden) was used to collect 1min fractions. Data was collected at 1 data point/sec on each of 2 wavelength channels (Waters 490 detector, Chan.1=215nm, Chan.2=280nm). All solvent programming was controlled by the Waters 840 module using 2 pumps. Samples were eluted using the following method:

Time (min)	Flow Rate (mL/min)	Buffer A: Buffer B
0 - 15	0.75	100:0 (isocratic)
15 - 17	0.75 - 1.5	100:0 (isocratic)
17 - 30	1.5	100:0 - 0:100
30 - 35	1.5	0:100 - 100:0

The column was reequilibrated at 100:0 (buffer A: buffer B) for 15min at 1.5mL/min before the next sample was injected.

Samples for separation by reversed-phase HPLC were prepared from cells (grown on Cytodex-3 microcarriers and harvested as described in section 4.2.8) as follows. The cells were suspended in 10mL G-buffer containing 1mM DTT, 10uM leupeptin and 0.125mM PMSF and lysed by sonication (as described in section 4.2.3) and left to stand for 10min on ice. All subsequent procedures were carried out at 4^{o}C. The nuclei and cell debris were pelleted by centrifugation (10min in a microfuge) and the pellets discarded. Cold acetonitrile was added dropwise to the supernatant, with stirring, to a total concentration of 30%(v/v) and the solution was then left for 60min. The resultant precipitate was pelleted by

centrifugation (12,000xg, 30min) and the supernatants and pellets were lyophilized and stored at -20°C. Prior to reversed-phase HPLC, the lyophilized supernatant was suspended in approximately 1mL of distilled water containing 0.1%(w/v) TFA. Insoluble material was removed by centrifugation (12,000xg, 30min) and the resultant supernatant injected onto the C4 reversed-phase column.

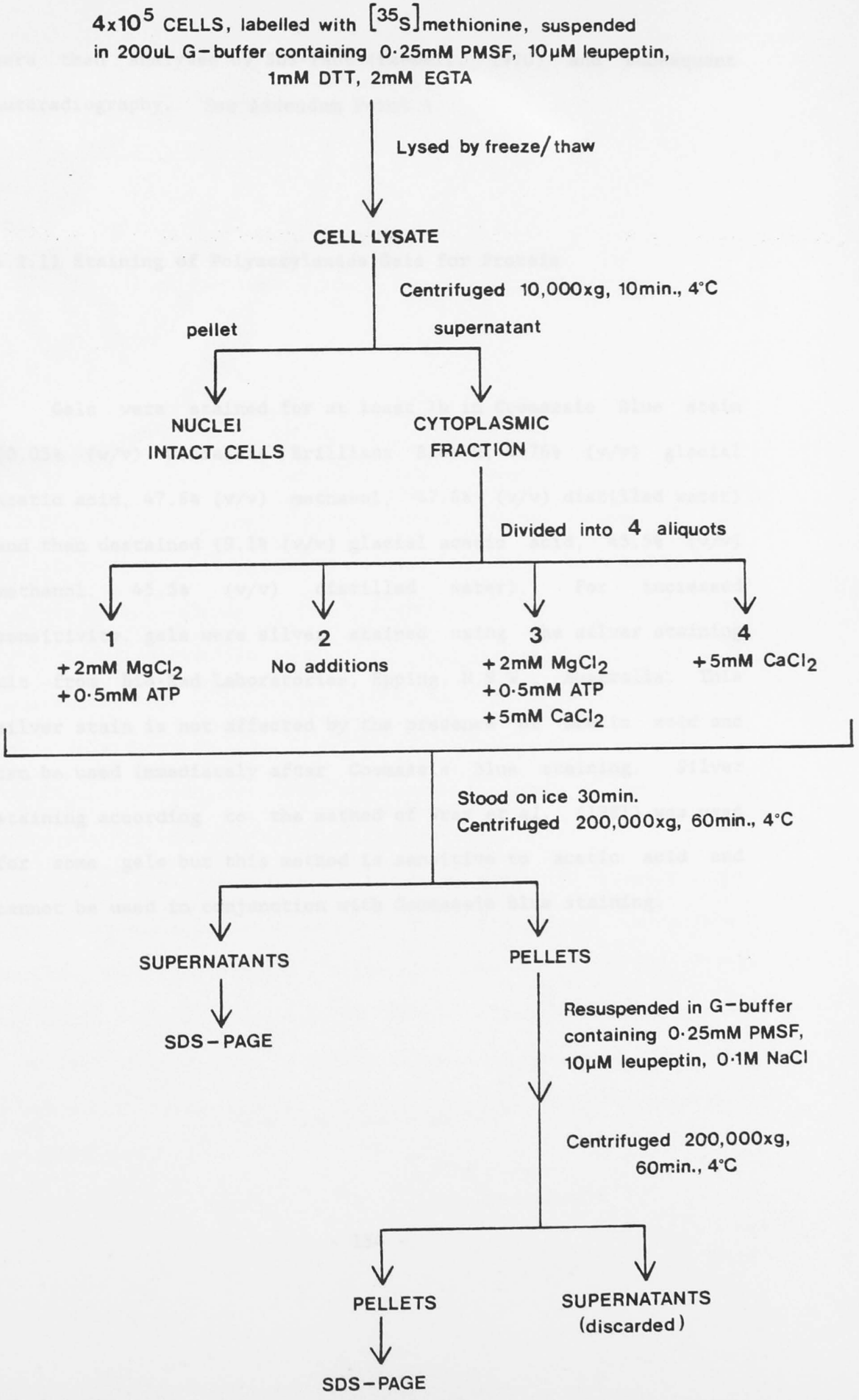
4.2.10 The F-Actin Pelleting Assay

Cells were plated in 5cm diameter petrie dishes ($4x10^5$ cells/dish) in DMEM supplemented with 5% (v/v) FCS, 5% (v/v) NCS, 2mM pyruvate and antibiotics (50 units/mL penicillin G, 50 units/mL streptomycin sulphate and 180 units/mL neomycin sulphate). After incubation for 24h at 37°C in a 5% CO_2/air humidified atmosphere, the dishes were rinsed with PBS and to each dish was added 5mL of methionine-free medium (see section 3.2) supplemented with 15% (v/v) DMEM (prepared as above), 2mM pyruvate, antibiotics (as above), 2mM glutamine and [^{35}S]methionine (approx. 35uCi). The cells were incubated overnight at 37°C in this medium and then each dish was rinsed (twice) with 5mL of PBS. The cells were detached from the petrie dishes by incubation in PBS containing 0.025% (w/v) trypsin and 0.02% (w/v) EDTA (1mL/petrie dish) for 10min at 37°C. The cells

were transferred to microfuge tubes, the dishes were rinsed with 200uL DMEM (supplemented as above) and the rinsings were then added to the microfuge tubes. Cells were pelleted by centrifugation (5s in a microfuge) and the supernatants were discarded. The cells were washed by suspension in 1mL PBS, pelleted (5s in a microfuge) and the supernatant discarded. The cells were then suspended (Fig. 16) in 200uL G-buffer containing 0.25mM PMSF, 10uM leupeptin, 1mM DTT and 2mM EGTA, frozen in dry ice, then thawed in water at 37°C. This freeze/thaw procedure was repeated twice to lyse the cells. The cell lysate was spun for 10min at 4°C in a microfuge to pellet nuclei, intact cells and cellular debris. The supernatant was transferred in 4x50uL aliquots to centrifuge tubes and supplemented with the following: (1) 2mM $MgCl_2$ and 0.5mM ATP, (2) no additions, (3) 2mM $MgCl_2$, 0.5mM ATP and 5mM $CaCl_2$, (4) 5mM $CaCl_2$. After incubating on ice for 30min, the solutions were centrifuged (200,000xg, 60min, 4°C), the supernatants aspirated and stored at -20°C and the pellets resuspended in G-buffer containing 0.25mM PMSF, 10uM leupeptin, 1mM DTT and 0.1M NaCl to wash away nonspecifically bound proteins. After centrifugation (200,000xg, 60min, 4°C) the supernatants were discarded and the pellets stored at -20°C.

The supernatants and pellets were subsequently thawed and to each was added 50uL electrophoresis sample buffer (10% (w/v) glycerol, 0.1% (w/v) bromophenol blue, 2% (w/v) SDS, 10mM Tris.HCl, pH 8.0, 0.1M DTT) before boiling for 5min. The samples

FIGURE 16: An outline of the F-actin pelleting assay procedure.


4x10^5 CELLS, labelled with [35S]methionine, suspended in 200uL G-buffer containing 0·25mM PMSF, 10μM leupeptin, 1mM DTT, 2mM EGTA
Lysed by freeze/thaw
CELL LYSATE
Centrifuged 10,000xg, 10min., 4°C
pellet
supernatant
NUCLEI INTACT CELLS
CYTOPLASMIC FRACTION
Divided into 4 aliquots
1
+2mM MgCl2
+0·5mM ATP
2
No additions
3
+2mM MgCl2
+0·5mM ATP
+5mM CaCl2
4
+5mM CaCl2
Stood on ice 30min.
Centrifuged 200,000xg, 60min., 4°C
SUPERNATANTS
SDS-PAGE
PELLETS
Resuspended in G-buffer containing 0·25mM PMSF, 10μM leupeptin, 0·1M NaCl
Centrifuged 200,000xg, 60min., 4°C
PELLETS
SDS-PAGE
SUPERNATANTS (discarded)

were then analysed by SDS-PAGE (Laemmli, 1970) and subsequent autoradiography. See Addendum Point 1

4.2.11 Staining of Polyacrylamide Gels for Protein

Gels were stained for at least 1h in Coomassie Blue stain (0.05% (w/v) Coomassie Brilliant Blue R, 4.76% (v/v) glacial acetic acid, 47.6% (v/v) methanol, 47.6% (v/v) distilled water) and then destained (9.1% (v/v) glacial acetic acid, 45.5% (v/v) methanol, 45.5% (v/v) distilled water). For increased sensitivity, gels were silver stained using the silver staining kit from Bio-Rad Laboratories, Epping, N.S.W., Australia. This silver stain is not affected by the presence of acetic acid and can be used immediately after Coomassie Blue staining. Silver staining according to the method of Wray *et al.* (1981) was used for some gels but this method is sensitive to acetic acid and cannot be used in conjunction with Coomassie Blue staining.

4.3 Results and Discussion

4.3.1 The Triton-Insoluble Pellet

The treatment of cells with nonionic detergents such as Triton X-100 results in the extraction of membrane lipids and the solubilization of some membrane-bound proteins. Some of the peripheral membrane proteins are insoluble under such conditions and can be concentrated by centrifugation. A large proportion of this insoluble fraction consists of cytoskeletal proteins and as a consequence, the cytoskeleton came to be defined as that portion of cellular protein insoluble in nonionic detergents. However, it should be stressed that there are many different forms of the cytoskeleton, each exhibiting different stabilities in non-ionic detergent. The method used in this study to prepare the Triton-insoluble pellet (section 4.2.2) differs from conventional methods used to prepare Triton-insoluble cytoskeletons. The method is designed to enrich for the microfilaments and associated proteins with the aim of making the protein composition of the preparation less complex than conventional Triton-insoluble cytoskeletons.

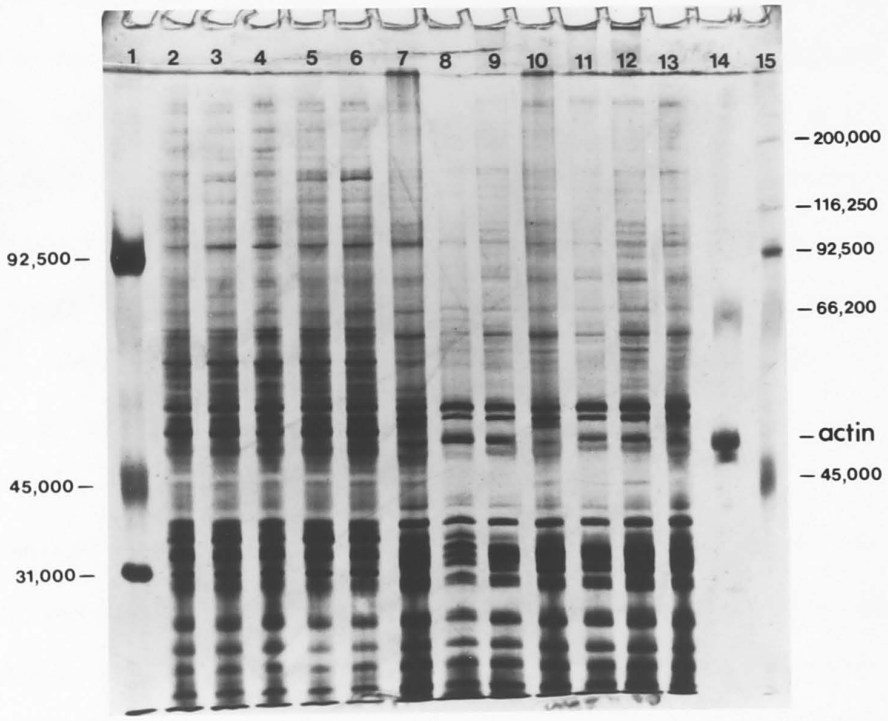
Cells were lysed and the cytoskeleton disrupted by homogenization then nuclei and large cellular fragments were removed by centrifugation. Actin polymerization was then induced by the addition of Mg^{2+} and KCl, and centrifugation of the resultant cytoplasmic gel concentrated filamentous actin, membrane vesicles and organelles (ribosomes, mitochondria) into a pellet. Extraction of this pellet with Triton X-100 buffer solubilizes membrane lipids and some membrane-bound proteins. Centrifugation concentrates the insoluble fraction which is enriched for microfilamentous material.

Analysis of the final Triton-insoluble pellet by SDS-PAGE shows that (Fig. 17), in comparison with the cell lysates, the Triton-insoluble pellets are enriched in a protein with an electrophoretic mobility identical to that of rabbit skeletal muscle actin. In addition, two proteins of about 50,000 M_r plus a number of proteins of 36,000 M_r and less are major components of the Triton-insoluble pellet, being present in approximately stoichiometric amounts, relative to actin.

While most actin-binding proteins would be expected to be present in concentrations considerably less than that of actin, the tropomyosins, which are F-actin-binding and stabilizing proteins, might be expected to be significantly concentrated in the Triton-insoluble pellet. The tropomyosins have molecular weights around 30,000 M_r and therefore probably constitute some of

FIGURE 17: Analysis of the cell lysates and Triton-insoluble pellets from several tumorigenic and nontumorigenic cell lines by SDS-PAGE.

The Triton-insoluble pellets were prepared as described in section 4.2.2. The protein content of lysates and Triton-insoluble pellets was determined using Folin phenol reagent method (Lowry *et al.*, 1951). Samples were applied to the 12% acrylamide gel at 30ug total protein per lane. Molecular weight standards were applied to lanes 1&15, and rabbit skeletal muscle actin to lane 14 (identification as indicated). The cell lysates were applied to lanes 2 to 6 and the Triton-insoluble pellets to lanes 7 to 13 with the cell lines being as follows: 5E (nontumorigenic), lanes 2&9; 5L (tumorigenic), lanes 3&10; 39E C13 (nontumorigenic), lanes 4&11; ESH 39 (tumorigenic), lanes 5&12; D98 AH2 (tumorigenic), lanes 6&13; HEp-2 (tumorigenic), lane 7; MRC-5 (nontumorigenic), lane 8.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
92,500
45,000
31,000
200,000
116,250
92,500
66,200
actin
45,000

the low molecular weight proteins in the Triton-insoluble pellet. In addition to the tropomyosins, the Triton-insoluble pellet would be expected to contain F-actin cross-linking, bundling and capping proteins as well as proteins involved in the attachment of microfilaments to cell membranes. All these proteins would be expected to be present in concentrations less than that of actin and would therefore be expected to be relatively minor components of the Triton-insoluble pellet. In general, these proteins have high molecular weights (60,000 M_r up to 270,000 M_r) and could constitute any of the relatively minor high molecular weight components of the Triton-insoluble pellets.

The method used to prepare the Triton-insoluble pellet is relatively non-specific and the pellet may contain, in addition to microfilament proteins, microtubule and intermediate filament proteins, as well as peripheral membrane proteins not associated wih the cytoskeleton, made insoluble by the extraction of lipids. Alpha- and beta-tubulin have molecular weights of 50,000 M_r and the cytokeratin and vimentin types of intermediate filaments, which might be expected to be present in the HeLa/fibroblast hybrid cells, have subunits of 40,000 M_r up to 70,000 M_r and around 56,000 M_r, respectively. It is possible that the major proteins of approximately 50,000 M_r and 60,000 M_r are tubulin or intermediate filament proteins. See Addendum Point 2

While there is some variation in the protein composition of

Triton-insoluble pellets from tumorigenic and nontumorigenic cells (Fig. 17), in general, no differences were detected that could be related to the tumorigenic phenotype. In particular, the pattern of low molecular weight proteins is apparently identical in the Triton-insoluble pellets for all cell lines. If the tropomyosins are indeed included in this group of low molecular weight proteins, then this result suggests that in the HeLa/fibroblast hybrid cell system, the re-expression of tumorigenicity is not associated with a reduction in tropomyosin content. Reductions in tropomyosin content, specifically the higher molecular weight isoforms, have been noted associated with malignant transformation in other cell systems (Lin *et al.*, 1985).

One protein with an electrophoretic mobility similar to that of the 45,000 M_r standard is present only in the Triton-insoluble pellet of tumorigenic cells (HEp-2, 5L, ESH 39 and, faintly, D98AH2; Fig. 17, lanes 7, 10, 12 & 13, respectively), but this difference could not be detected in subsequent analyses and, because the protein is present in very small amounts, the significance of this difference is doubtful. It can, therefore, be concluded that, at the level of resolution provided by SDS-PAGE, the Triton-insoluble pellets of tumorigenic cells are not significantly different from the pellets of the nontumorigenic cells. This suggests that the expression of actin cross-linking and bundling proteins, F-actin-binding proteins, and membrane-associated actin-binding proteins is similar in the

tumorigenic and nontumorigenic cells.

While this conclusion requires further investigation, the Triton-insoluble pellet is a less than ideal system for further analysis. The complexity of the protein composition and the low degree of specificity of the technique towards the selection of actin-binding proteins might be able to be overcome but, in addition, the Triton-insoluble pellet is difficult to resuspend during the washing steps. This difficulty was exacerbated by the small size of the pellet obtained for these analytical-scale preparations. Hence, it is possible that some of the minor components of the pellets are due to carryover resulting from incomplete washing of the pellets. For any further analysis of this pool of actin-binding proteins an alternative technique would be preferable.

4.3.2 Actin Monomer-Binding Proteins

4.3.2.1 Enrichment of Actin Monomer-Binding Proteins by DNase I-Affinity Chromatography

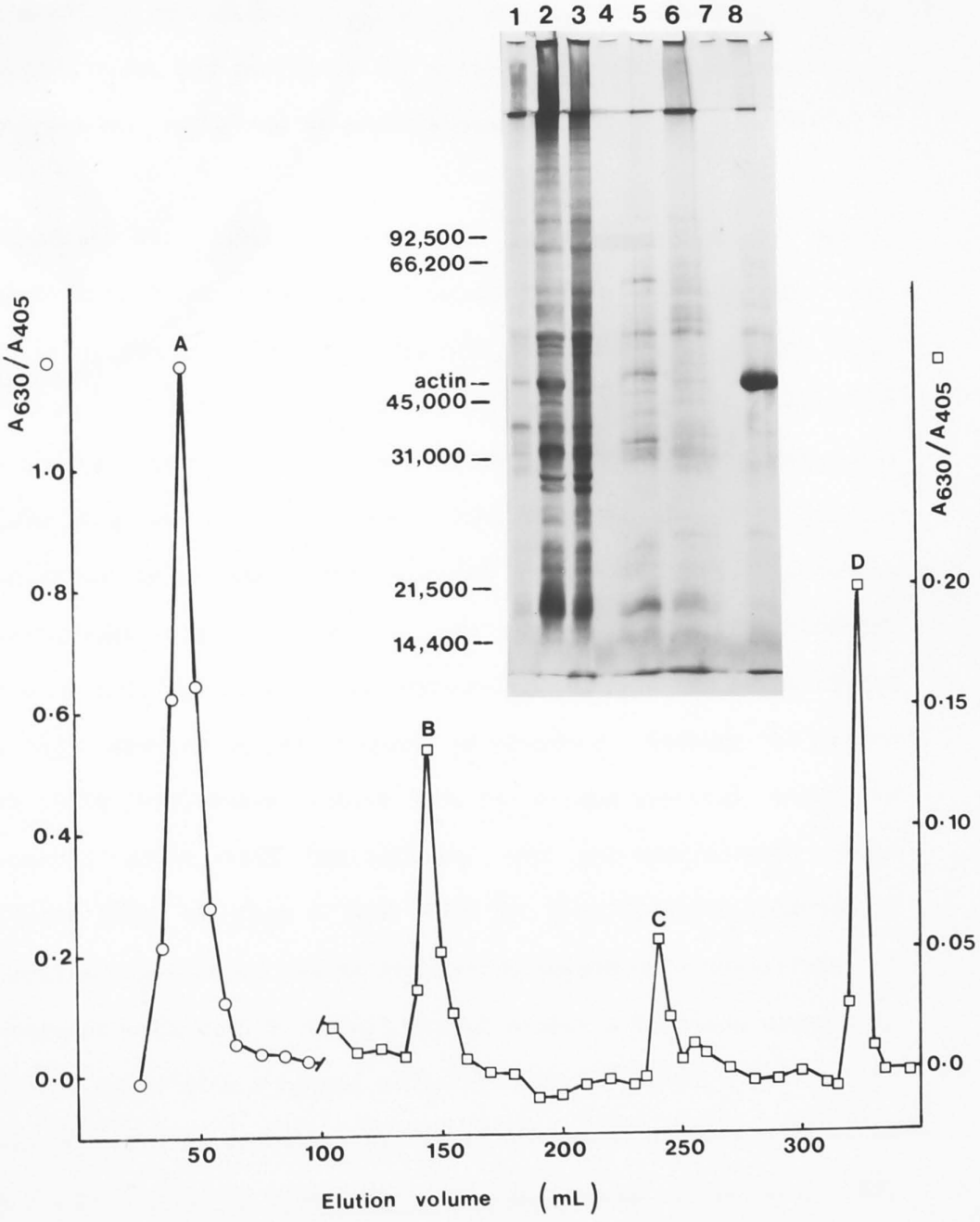
Monomeric actin binds to DNase I with high affinity, and consequently monomeric actin will be adsorbed to a column of DNase I-linked Sepharose. In addition, any proteins which bind to monomeric actin on sites distinct from the DNase I-binding site will also be retained. The specificity and strength of these interactions enables DNase I-affinity chromatography to be used to rapidly purify actin monomer-binding proteins from crude cell extracts.

Figure 18 shows a typical elution profile for the DNase I-Sepharose column with SDS-PAGE analysis of the pooled peak fractions. This profile is for a nontumorigenic cell line, 5E, but other profiles were almost identical. The column eluate was monitored by absorbance at 280nm and, while the elution profile obtained in this way closely resembles the profile shown in figure 18, there are substantial shifts in absorbance associated with the buffer changes. For clarity, and to confirm the selection of

FIGURE 18: A typical elution profile for the purification of actin monomer-binding proteins by DNase I-affinity chromatography.

The cells (5E, 3.2×10^7) were lysed by sonication and the nuclei removed by centrifugation (Lane 1). The lysate supernatant (Lane 2) was applied to the DNase I-Sepharose column and washed through with sonication buffer. Proteins not retained by the column were eluted as the flow-through (Peak A and Lane 3). Proteins retained with low affinity were eluted with 0.1M NaCl buffer (Peak B and Lane 5) before proteins bound with higher affinity were eluted with 0.6M NaCl buffer (Peak C and Lane 6). Finally actin monomer was dissociated from the DNase I ligand, along with remaining actin-binding proteins, using 3M Gu.HCl buffer (peak D and lane 8).

Note that the leading edge of each peak of eluted material follows closely the buffer interface that results from the changes in column buffer.


1 2 3 4 5 6 7 8
92,500
66,200
actin
45,000
31,000
21,500
14,400
A630/A405
A630/A405
A
B
C
D
1·0
0·8
0·6
0·4
0·2
0·0
0·20
0·15
0·10
0·05
0·0
50
100
150
200
250
300
Elution volume (mL)

important fractions, samples of each fraction were assayed for protein content by the Micro-Bradford method. In some instances the resultant absorbance (A_{630}/A_{405}) was outside the range of the standard curve and therefore the profile is plotted as absorbance ratio and not converted to protein content.

After cell lysis, the lysate was centrifuged to remove nuclei, intact cells and large fragments of cellular debris. Less than 1% of the total actin is present in this nuclear pellet (Fig. 18, Lane 1) indicating that virtually all the actin passes into the lysate supernatant that is applied to the DNase I-Sepharose column (Fig. 18, Lane 2). More than 95% of the total protein applied to the column is not retained but washes through into the flow-through (Fig. 18, Lane 3 and Peak A). The flow-through contains only traces of actin indicating that greater than 95% of the actin applied to the column is retained. Washing the column with 0.1M NaCl buffer elutes from the column proteins bound to monomeric actin with low affinity and non-specifically bound proteins (Fig. 18, Lane 5 and Peak B). This fraction constitutes approximately 1.5% of the total protein loaded onto the column. A subsequent wash with 0.6M NaCl buffer elutes a fraction containing proteins bound with a higher affinity (Fig. 18, lane 6 and Peak C) which constitutes approximately 1% of the total protein applied to the column. Finally, treatment of the column with 3M Gu.HCl dissociates the actin monomer from the DNase I ligand along with any remaining tightly bound proteins (Fig. 18, Lane 8 and Peak D).

This fraction constitutes 2% of the total protein applied to the column. The major protein in the Gu.HCl fraction is actin but 5 or 6 minor components are also apparent.

DNase I-affinity chromatography was used to prepare fractions enriched in actin-binding proteins from four of the HeLa/fibroblast hybrid cell lines, the D98 AH2 HeLa parental cell line as well as primary human fibroblasts (A164, obtained from amniocentesis samples). Figure 19a and 19b show the SDS-PAGE analysis of these fractions. Note that the gel shown in figure 19a is 10% in acrylamide and the gel in figure 19b is 12%. While comparison between the two gels is possible, the 10% gel does not resolve low molecular weight proteins.

The protein composition of each of the 0.1M NaCl wash, the 0,6M NaCl wash and the Gu.HCl eluate varied very little with cell line. There are some differences but none that can be correlated with the tumorigenic phenotype. However the consistency of protein composition does indicate the high degree of reproducibility of the technique.

Bands of similar electrophoretic mobility are present in more than one fraction. For example, bands with an electrophoretic mobility corresponding to 50,000 M_r are present in both the 0.6M NaCl wash and the 0.1M NaCl wash. This suggests that, rather than being quantitatively eluted in a single fraction, some proteins

FIGURE 19a: Analysis by SDS-PAGE of DNase I-affinity chromatography fractions from primary fibroblasts (A164) and the D98 AH2 HeLa cells.

DNase I-affinity chromatography was carried out as described in section 4.2.3. The lyophilized fractions were solubilized in distilled water and the protein content determined by the Micro-Bradford method. Samples were applied to a 10% acrylamide gel at approximately 12ug total protein per lane. Molecular weight standards were applied to lanes 1&19, and rabbit skeletal muscle actin to lane 2. Fractions from the A164 fibroblasts were applied to lanes 4 to 6 and 8 to 10 while fractions from the D98 AH2 HeLa cells were applied to lanes 12, 13 and 15 to 17. The fractions used were as follows: cell lysate (lanes 4&12); nuclear pellet (lane 5, A164 only); flow-through (lanes 6&13); 0.1M NaCl wash (lanes 8&15); 0.6M NaCl wash (lanes 9&16); Gu.HCl eluate (lanes 10&17).

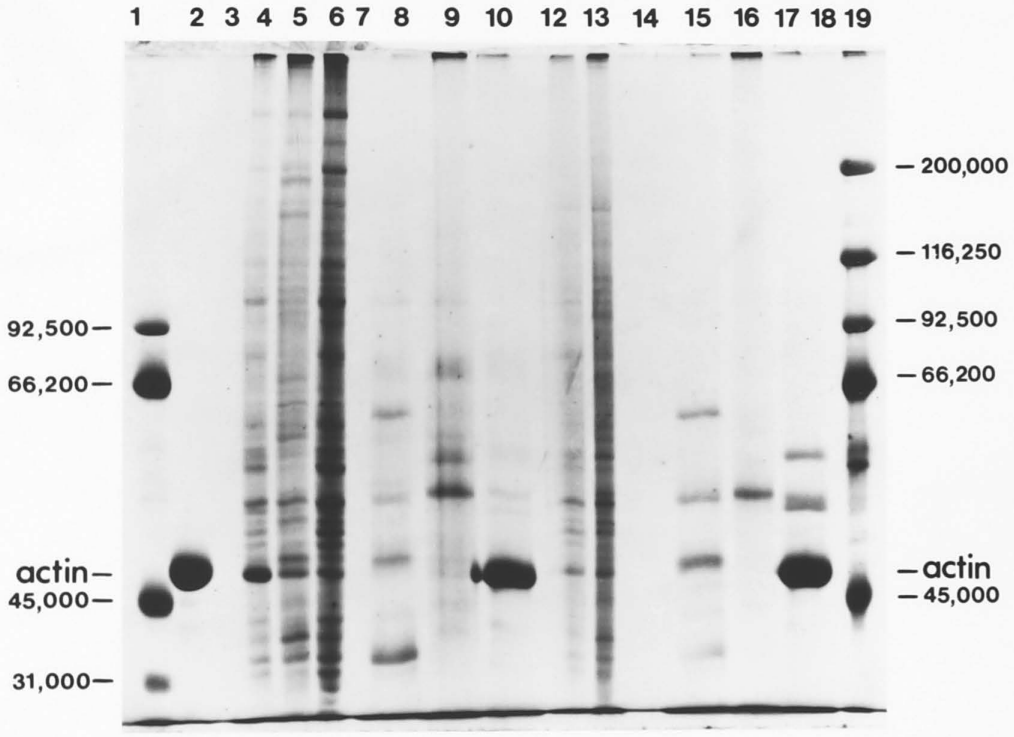
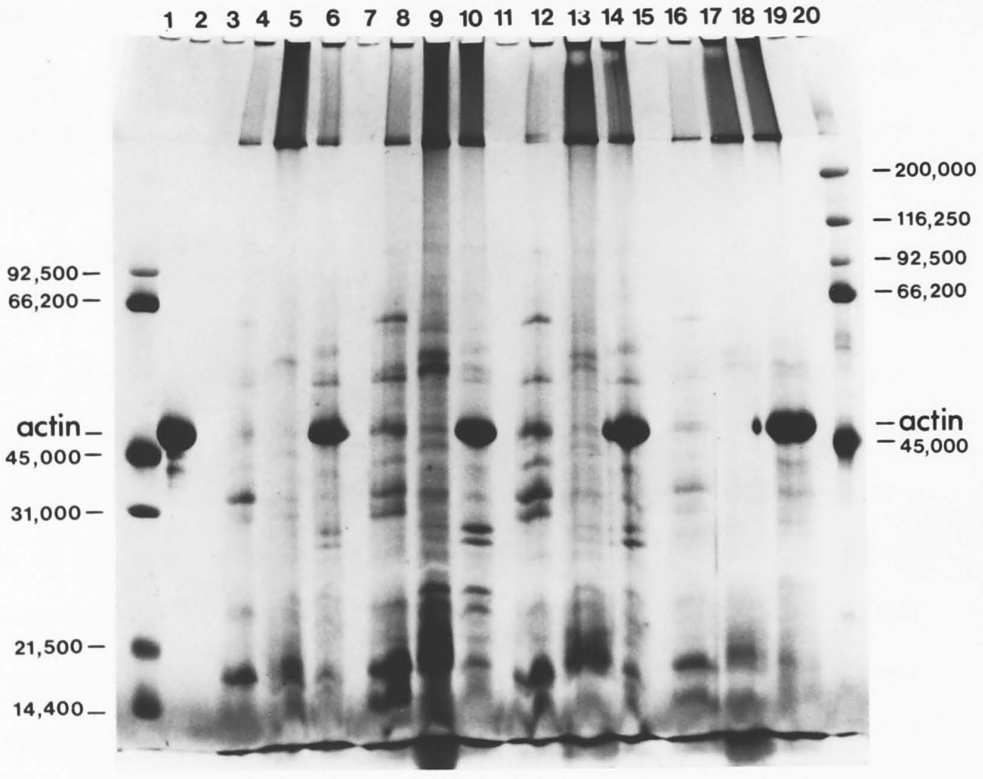

1 2 3 4 5 6 7 8 9 10 12 13 14 15 16 17 18 19
92,500
66,200
actin
45,000
31,000
200,000
116,250
92,500
66,200
actin
45,000

FIGURE 19b: Analysis by SDS-PAGE of DNase I-affinity chromatography fractions from four HeLa/fibroblast hybrid cell lines.

DNase I-affinity chromatography was carried out as described in section 4.2.3. The lyophilized fractions were solubilized in distilled water and the protein content determined by the Micro-Bradford method. Samples were applied to a 12% acrylamide gel at approximately 6ug total protein per lane. Molecular weight standards were applied to lanes 1&20, and rabbit skeletal muscle actin to lane 2. The fractions used were from the following cell lines: 5L (tumorigenic), lanes 4 to 6; 5E (nontumorigenic), lanes 8 to 10; IA3 cn TG (tumorigenic), lanes 12 to 14; IA3 cn 2.1 (nontumorigenic), lanes 16 to 18. The fractions are as follows: 0.1M NaCl wash, lanes 4, 8, 12 & 16; 0.6M NaCl wash, lanes 5, 9, 13 &17; Gu.HCl eluate, lanes 6, 10, 14 & 18.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
92,500
66,200
actin
45,000
31,000
21,500
14,400
200,000
116,250
92,500
66,200
actin
45,000

may be partially eluted in more than one fraction. The DNase I-affinity chromatography method elutes proteins from the column according to the affinity with which they bind to monomeric actin. Elution in more than one fraction would imply that the cellular pool of that actin-binding protein is heterogeneous in terms of the affinity with which it binds to actin. Such heterogeneity may result from the presence of isoforms with differing affinities for actin or as a result of modifications, such as phosphorylation, of the protein(s) concerned.

For example, it has been reported that a 33,000 M_r isoform of tropomyosin binds to actin filaments with higher affinity than do the 30,000 and 32,000 M_r isoforms (Keiser & Wegner, 1985). As a result of this differing affinity, the presence of the 55,000 M_r bundling protein of HeLa cells causes the low (32,400 and 32,000) M_r tropomyosins to be dissociated from F-actin to a greater extent than are the high (40,000 and 36,500) M_r tropomyosins (Matsumura & Yamashiro-Matsumura, 1986). Alpha-actinin of platelets has also been demonstrated to exist as isoforms differing in chain length, immunological cross-reactivity and sensitivity to a Ca^{2+}-dependent protease as well as in the sensitivity of F-actin-binding activity to Ca^{2+} and the efficiency of cross-linking (Landon *et al.*, 1985).

While F-actin binding proteins such as the tropomyosins would not be expected to be present in DNase I-affinity purified fractions, it is possible that actin monomer-binding proteins

might also exhibit isoforms with differing affinities for actin. Similarly, although there are as yet no actin monomer-binding proteins for which it has been demonstrated that phosphorylation reduces the affinity of binding to actin, the phosphorylation of MAP-2 may reduce the affinity with which MAP-2 binds to F-actin (Akiyama *et al.*, 1986; Sattilaro, 1986). An increased proportion of high- or low-affinity forms of actin-binding proteins might alter the overall actin organization through alterations in the competition for binding sites on the actin molecule or through alterations in the sizes of the pools of actin monomer and polymer. Hence it would be of interest to quantitate the amounts of actin-binding proteins in the different DNase I-affinity chromatography fractions from tumorigenic and nontumorigenic cells.

Quantitation of the amount of actin-binding proteins in the various fractions was attempted by loading samples onto the gels, according to determinations of total protein content, to give the same amount of total protein in each lane. Hence differences in the intensity with which a band is stained in different fractions might be expected to indicate differences in the content of that protein, relative to total protein in the fraction. For instance, the minor components of the Gu.HCl eluate with molecular weights of 55,000 and 48,000 M_r appear to be present in greater amounts in the D98 AH2 Gu.HCl eluate (Fig. 19a, Lane 17) than in the A164 fibroblast Gu.HCl eluate (Fig. 19a, Lane 10). However, for a number of technical reasons, determination of the protein content of each sample was associated with a high degree of

error. Indeed, examination of the intensity of staining suggests considerable variation in total protein per lane (for example compare Fig. 19b, Lane 9 with Lane 17).

The fractions from the DNase I-Sepharose column were dialysed against 0.05M NH_4CO_3 to remove salts, then concentrated by lyophilization. The lyophilized material was not completely soluble in low ionic strength buffers and the sample buffer used for SDS-PAGE. The solubility problems were most marked with the Gu.HCl eluate as a result of the denaturation of actin by Gu.HCl. Consequently, the degree with which actin is solubilized may be variable and the ratio of actin to other proteins seen on the gel may reflect actin solubility and not the level of actin-binding proteins in the cell. As a consequence of these problems, little emphasis can be placed on any apparent differences in quantity of individual proteins in the DNase I-affinity chromatography fractions.

A further consequence of the incomplete solubility of the lyophilized fractions, possibly in combination with some residual salts, is the presence of streaking artifacts in the gels resulting in a reduced resolution. It also appears that some material does not penetrate from the stacking gel into the running gel. However, this was only visible when the protein bands in the gel were visualized using the Bio-Rad silver stain and was not apparent when gels were stained by the Coomassie Blue method. The

lyophilized material is readily solubilized with high ionic strength buffers, such as 250mM NaCl, 10mM Tris.HCl, pH 8.0, and where possible this buffer was used for solubilization.

While no definite identifications can be made, it is worth considering those actin-binding proteins that may be purified by DNase I-chromatography. In general, the proteins retained on the column will be actin monomer-binding proteins. F-actin-binding proteins may bind to actin monomer but with relatively low affinity and such proteins would be expected to be eluted by the 0.1M NaCl wash. It is noticeable that high molecular weight proteins are not detectable in any of the 0.1M NaCl, 0.6M NaCl or Gu.HCl fractions and, in general, the high molecular weight actin-binding proteins are F-actin cross-linking and bundling proteins. High molecular weight proteins are present in the flow-through (Fig. 19a, Lane 6) suggesting that these proteins are indeed not retained by the DNase I-Sepharose column. It must also be considered that proteins other than monomeric actin, that bind to DNase I will be retained on the column. An F-actin capping protein from *Physarum*, Cap 42(a+b), will bind directly to DNase I (Maruta & Isenberg, 1984). However, binding to DNase I can be attributed to the Cap 42(b) subunit which is structurally similar to actin although it does not polymerize.

A number of low molecular weight proteins have been purified from a variety of cell types using DNase I-affinity

chromatography. Depactin, a 17,000 M_r actin depolymerizing protein from starfish oocytes, binds to DNase I-Sepharose via actin and is eluted with 0.6M KCl or 0.6M KI (Mabuchi, 1983). Low molecular weight (18,000, 19,000 and 26,000 M_r) actin depolymerizing proteins have also been purified by DNase I-affinity chromatography from porcine kidney and brain (Maekawa *et al.*, 1984; Nishida *et al.*, 1985) as well as from ascites hepatoma cells (Ohta *et al.*, 1984). In addition, actophorin, a 15,000 M_r actin-severing protein from *Acanthamoeba castellanii*, binds to actin monomer (Cooper *et al.*, 1986) and might also be expected to bind to DNase I-Sepharose via actin. Profilin is known to bind to DNase I-agarose and is eluted with 2M urea (Maekawa *et al.*, 1984) but not by high ionic strength. It seems possible, therefore, that proteins with apparent molecular weights in the region of 15,000 to 20,000 M_r eluted in the 0.1M NaCl wash, the 0.6M NaCl wash and the Gu.HCl eluate may be low molecular weight actin depolymerizing proteins or profilin.

Gelsolin, a 90,000 M_r protein with actin filament capping, severing and nucleating activities in the presence of calcium, has been purified by DNase I-affinity chromatography from platelets (Markey *et al.*, 1982; Kurth & Bryan, 1984; Olomucki *et al.*, 1984), from bovine aorta (Isenberg *et al.*, 1983; Kanno *et al.*, 1985) and from a hamster cell line (Nelson & Boyd, 1985). In the presence of calcium, gelsolin binds to monomeric actin with high affinity.

The binding is stable under conditions of high ionic strength but chelation of calcium with EGTA will elute gelsolin from a DNase I-affinity column. However, bands of 90,000 M_r are not apparent in any of the 0.1M NaCl washes, the 0.6M NaCl washes or the Gu.HCl eluates for the cell lines used here for DNase I-affinity chromatography. This suggests that either the cell lines used contain insufficient gelsolin for it to be detected by SDS-PAGE or the properties of gelsolin in these cells are such that it is not retained on the DNase I-Sepharose column under the conditions used.

Fragmin, a 42,000 M_r capping protein from *Physarum*, forms a high affinity complex with actin in the presence of calcium (Maruta *et al.*, 1984) and would be expected to bind to DNase I-Sepharose. SU45, a 45,000 M_r protein with properties similar to fragmin has been isolated by DNase I-affinity chromatography as a 1:1 complex with actin from unfertilized sea urchin eggs (Hosoya & Mabuchi, 1984). The Gu.HCl eluate contains a protein of 48,000 M_r which could be related to fragmin or SU45.

It has been demonstrated with a ^{125}I-actin overlay technique that two glycolytic enzymes, glyceraldehyde-3-phosphate dehydrogenase (36,000 M_r) and aldolase (40,000 M_r) bind to actin and binding is reduced but not eliminated by 1.0M NaCl (Snabes *et al.*, 1983). Thus it is possible that proteins with molecular weights in the region of 15,000 to 20,000 M_r eluted in the 0.1M

NaCl wash, the 0.6M NaCl wash and the Gu.HCl eluate are glycolytic enzymes.

It is concluded from the comparison of the protein compositions of DNase I-affinity chromatography fractions from tumorigenic and nontumorigenic HeLa/fibroblast hybrid cells that the pattern of actin monomer-binding proteins present in the tumorigenic cells is not significantly different from that of the nontumorigenic cells. Actin monomer-binding proteins possibly present in the DNase I-affinity chromatograpy fractions include profilin, low molecular weight depolymerizing proteins and barbed-end capping proteins, possibly related to fragmin.

4.3.2.2 Assays of Actin-Binding Protein Activity in the DNase I-Affinity Chromatography Fractions

Given that the proteins enriched by DNase I-affinity chromatography are mainly actin-binding proteins, it is of interest to characterize the activities present in the column eluate and to quantitate their relative activities in tumorigenic and nontumorigenic cells. The assays of actin-binding protein activity were carried out using the techniques of falling-ball viscometry and pyrene-actin fluorescence enhancement.

Falling-ball (low shear) viscometry measures the viscosity of solutions as the time taken for a ball-bearing to fall a distance through the solution contained in a vertical capillary tube. The viscosity of solutions of pure F-actin is dependent on the length of the actin filaments, entanglement of the filaments, and weak interactions between the filaments. The presence of actin cross-linking proteins, in appropriate concentrations, can result in the formation of a gel thereby increasing the viscosity above that of pure F-actin solutions. Similarly, the presence of F-actin capping or severing proteins will result in the formation of a large number of short actin filaments and a viscosity less than that of pure F-actin solutions. Thus falling-ball viscometry can be used to provide information on the effect of actin-binding proteins on the organization of actin filaments.

The incorporation of pyrene-labelled actin into actin polymer is associated with a considerable enhancement of fluorescence. The technique of pyrene-actin fluorescence enhancement is therefore appropriate for following the kinetics of actin polymerization both in the presence and absence of actin-binding proteins.

Falling-ball viscometry was used to assess the viscosity attained by F-actin solutions in the presence of the different fractions produced during DNase I-affinity chromatography (Table

TABLE 18: The effect of cell lysate and DNase I-affinity chromatography fractions on the viscosity of F-actin solutions, as determined by falling-ball viscometry.

Sample	Total protein (ug)	Viscosity
control	-	infinity
cell lysate	3	infinity
	6	1min 55s
	10	>2min
	50	1s
flow-thru'	3	infinity
	6	infinity
	10	>2min
	50	12s
0.1M NaCl wash	3	1min 38s
	5.2	7s
0.6M NaCl wash	3	infinity
	6	41s
Gu.HCl eluate	3	1min 38s
	6	8s

Viscosity is assessed as the time taken for a ball bearing to fall 12cm through the actin solution, contained in a vertical capillary tube. A value of infinity is given for any time >4min. The cell line used for this experiment was the nontumorigenic HeLa/fibroblast hybrid cell line, IA3 cn 2.1 and using 100ug actin in 400uL of assay solution. Viscosity measurements were made 30min after the induction of actin polymerization. The viscosity of water controls was measured as 0.8s. The protein content of the samples was determined by the Micro-Bradford method.

18). While the cell lysate applied to the column and the flow-through both cause a reduction in actin viscosity, the specific activity is low. The 0.1M NaCl wash, 0.6M NaCl wash and Gu.HCl eluate all cause a reduction in actin viscosity at a much lower concentration of protein indicating enrichment of actin-binding protein(s) in these fractions, relative to the cell lysate.

While it can not be determined by falling-ball viscometry whether the activities in the 0.1M NaCl wash, 0.6M NaCl wash and Gu.HCl eluate are due to similar or different actin-binding proteins, in all three fractions the activity is detected at protein concentrations substoichiometric relative to actin. The falling-ball viscometry assays were carried out using actin concentrations of 100ug/400uL and a reduction in actin viscosity was detected at sample protein concentrations of 6ug/400uL. Even if the activity is that of a low molecular weight protein or the combined activity of more than one protein, the stoichiometry will still be considerably less than one.

The effect of calcium on the actin-binding protein activities in the 0.1M and 0.6M NaCl washes as well as the Gu.HCl eluates was examined by falling-ball viscometry in the presence of 100uM Ca^{2+} or 100uM $CaCl_2$ and 5mM EGTA (Table 19). Interpretation of these results is complicated by a direct effect of Ca^{2+} on actin polymerization: 30min after the initiation of actin

TABLE 19: The effect of DNase I-affinity chromatography fractions on the viscosity of F-actin solutions in the presence and absence of calcium.

Sample	Viscosity	
	$+Ca^{2+}$	$-Ca^{2+}$
Control	1min 33s	3min 24s
D98 AH2		
0.1M NaCl wash	1s	1s
0.6M NaCl wash	1s	1s
Gu.HCl eluate	38s	46s
IA3 cn 2.1		
0.1M NaCl wash	1s	1s
0.6M NaCl wash	1s	-
Gu.HCl eluate	16s	34s
IA3 cn TG		
0.1M NaCl wash	18s	8s
0.6M NaCl wash	2s	2s
Gu.HCl eluate	1min 30s	43s
5L		
0.1M NaCl wash	1min 17s	40s
0.6M NaCl wash	13s	13s
Gu.HCl eluate	infinity	infinity
5E		
0.1M NaCl wash	6s	10s
0.6M NaCl wash	2s	3s
Gu.HCl eluate	3s	8s

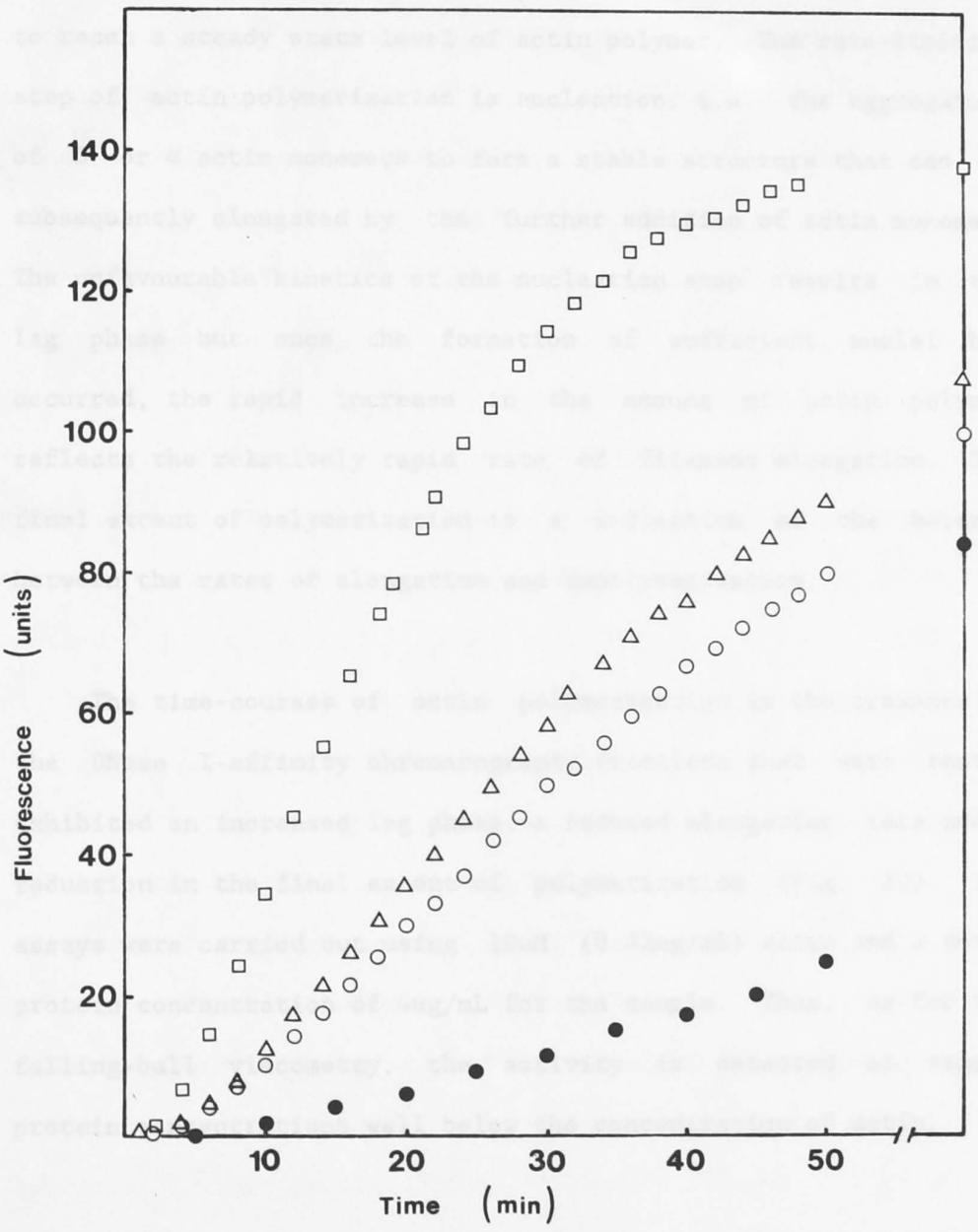
Viscosity was assessed as described in the footnote to table 18. Measurements were made 30min after the induction of actin polymerization with samples added at a total protein concentration of approximately 3.5ug/mL assay solution. Actin concentrations were 225ug/mL assay solution.

polymerization, the viscosity of F-actin solutions is greater in the absence of calcium than it is in the presence of calcium. Allowing for this difference, the data shown in table 19 indicate that the actin-binding protein(s) causing the reduction in F-actin viscosity is (are) probably not sensitive to calcium. In an attempt to verify this result, falling-ball viscometry was used to assess the effect of the 0.6M NaCl wash fractions from the 5E and 5L cell lines on the viscosity of F-actin solutions in the presence of various concentrations of free calcium (data not shown). However, the effect of calcium on the viscosity of actin controls was such that under conditions of very low free calcium concentrations, viscosities were high and consequently difficult to measure with any accuracy. In addition, the amount of sample available for use in the assay was limited and only one assay was carried out at each calcium concentration. This added source of error made it impossible to draw conclusions from the data with any degree of certainty.

To obtain more information on the type of actin-binding protein activity present in the DNase I-affinity purified fractions, the pyrene-actin fluorescence enhancement method was used to follow actin polymerization in the presence of the 0.6M NaCl wash and Gu.HCl eluate fractions (Fig. 20). The control curve shows a characteristic time-course for the Mg^{2+}-induced polymerization of actin from monomer in the absence of actin-binding proteins. The time-course typically exhibits an

FIGURE 20: Time courses of actin polymerization from actin monomer, determined by the pyrene-actin fluorescence enhancement method.

Details of the method are given in section 4.2.5. The polymerization of pure actin monomer (0.42mg/mL or 10uM), induced by 2mM Mg^{2+}, was used as a control time course (□). Time courses of actin polymerization were followed in the presence of DNase I-affinity chromatography fractions using total protein concentrations of 4ug/mL. The following fractions were used: (△) 0.6M NaCl wash from 5E (nontumorigenic) cells; (○) Gu.HCl eluate and (●) 0.6M NaCl wash from 5L (tumorigenic) cells.


140
120
100
80
60
40
20
Fluorescence (units)
10
20
30
40
50
Time (min)

initial lag phase, followed by a period of rapid polymerization, to reach a steady state level of actin polymer. The rate-limiting step of actin polymerization is nucleation, i.e. the aggregation of 3 or 4 actin monomers to form a stable structure that can be subsequently elongated by the further addition of actin monomer. The unfavourable kinetics of the nucleation step results in the lag phase but once the formation of sufficient nuclei has occurred, the rapid increase in the amount of actin polymer reflects the relatively rapid rate of filament elongation. The final extent of polymerization is a reflection of the balance between the rates of elongation and depolymerization.

The time-courses of actin polymerization in the presence of the DNase I-affinity chromatography fractions that were tested exhibited an increased lag phase, a reduced elongation rate and a reduction in the final extent of polymerization (Fig. 20). The assays were carried out using 10uM (0.42mg/mL) actin and a total protein concentration of 4ug/mL for the sample. Thus, as for the falling-ball viscometry, the activity is detected at sample protein concentrations well below the concentration of actin.

It can be concluded that the activity enriched in fractions produced by DNase I-affinity chromatography, when present in low concentrations relative to actin, causes a reduction in the viscosity of F-actin solutions, reduces the rates of nucleation and elongation, and reduces the final extent of actin

polymerization. These activities are probably not affected by the concentration of calcium but this requires verification. In section 4.3.2.1, the possible identification of proteins in the DNase I-affinity column fractions was considered on the basis of molecular weight and actin-binding properties. Many of these proteins exhibit characteristics that could explain the observed activity towards actin.

The activity is, in general, typical of a barbed-end capping protein. One protein which exhibits similar activity is SU45, the 45,000 M_r protein from sea urchin eggs which is suggested to be a barbed end capping protein (Hosoya & Mabuchi, 1984). SU45 was isolated as a 1:1 complex with actin and the complex will inhibit the initial rate of actin polymerization, inhibit the annealing to F-actin fragments, prevent actin filaments from depolymerizing and inhibit the addition of actin monomer at the barbed end of filaments in addition to causing the final viscosity of F-actin solutions to be reduced. At relatively high, but still substoichiometric, molar ratios of complex to actin, the rate of nucleation is accelerated. All of these activities occur in the presence and absence of calcium (Hosoya & Mabuchi, 1984). In the pyrene-actin assays of polymerization in the presence of DNase I-affinity purified fractions from HeLa/fibroblast cells, no nucleating activity was detected but this may have been due to the use of protein concentrations too low for detection of enhanced nucleation. Fragmin, the 42,000 M_r protein from *Physarum*

plasmodium has similar properties to SU45 with the exception that fragmin requires the presence of calcium for activity (Hatano *et al.*, 1982).

The low molecular weight actin depolymerizing proteins are also calcium-independent in their activity. Actophorin is a 15,000 M_r protein from *Acanthamoeba castellanii* that is present in the cell at a molar ratio of ~10:1, actin to actophorin. Actophorin reduces the extent of actin polymerization in a concentration-dependent manner and forms a nonpolymerizable complex with pyrene-actin. Actophorin inhibits elongation at both ends of actin filaments and while low concentrations decrease the length and low shear viscosity of actin filaments, high concentrations cause preformed actin filaments to shorten rapidly (Cooper *et al.*, 1986). Depactin, a 17,000 M_r protein from starfish oocytes, also reduces the extent of actin polymerization and increases the apparent critical concentration for polymerization (Mabuchi, 1983). Depactin would be expected to be eluted in the 0.6M NaCl wash. Profilin prolongs the lag phase and inhibits the rate and extent of polymerization in a concentration-dependent manner and the presence of a barbed-end capping protein potentiates the inhibitory action of profilin (Isenberg *et al.*, 1983). Thus it is possible that the apparent activities result from the actions of more than one protein in each of the fractions.

Limited proteolysis of gelsolin produces proteolytic domains which retain the ability to form 1:1 complexes with actin monomer thereby inhibiting polymerization (Kwiatkowski *et al.*, 1985). The presence of these fragments could also explain the observed activities. No proteins with molecular weights similar to gelsolin (90,000 M_r) were detected in the fractions tested and proteolysis should have been inhibited by the presence of PMSF and leupeptin. However, to confirm this all three major fractions (0.1M NaCl wash, 0.6M NaCl wash and Gu.HCl eluate) were separated by SDS-PAGE then blotted with anti-gelsolin antibody as described in section 4.2.6. The only reactive band was the positive control. Therefore neither gelsolin, gelsolin-related proteins, nor proteolytic fragments of gelsolin are present in the column eluate. Banyard *et al.* (personal communication) have shown by Western blotting using the same gelsolin antibody, that the HeLa/fibroblast hybrid cells and the D98 AH2 HeLa parent cells do contain gelsolin but the gelsolin content of these cells is considerably less (by a factor of 10) than the gelsolin content of primary fibroblasts. The absence of detectable gelsolin in the DNase I-affinity purified material indicates that either gelsolin content is below the limits of detection of the methods used or, unlike platelet gelsolin, the gelsolin of fibroblasts and epithelial cells is not adsorbed to DNase I-Sepharose via actin monomer under the conditions used.

The pyrene-actin fluorescence assays of actin polymerization

in the presence of DNase I-affinity purified material (Fig. 20) suggests that the postulated barbed-end capping activity may be present in greater amounts in the 0.6M NaCl wash from 5L cells than the 0.6M NaCl wash from 5E cells. The same concentration of sample protein (4ug/mL) was used for the two assays indicating that the specific activity is greater in the fraction from 5L cells. Such a difference in capping/severing activity could be of significance to the disorganization of microfilaments in tumorigenic cells. To further investigate this possible quantitative difference in activity in fractions from tumorigenic and nontumorigenic cells, falling-ball viscometry was used to prepare a dose-response curve for each of the 0.1M NaCl washes, 0.6M NaCl washes and the Gu.HCl eluates from the 5E and 5L cell lines (Fig. 21). See Addendum Point 3

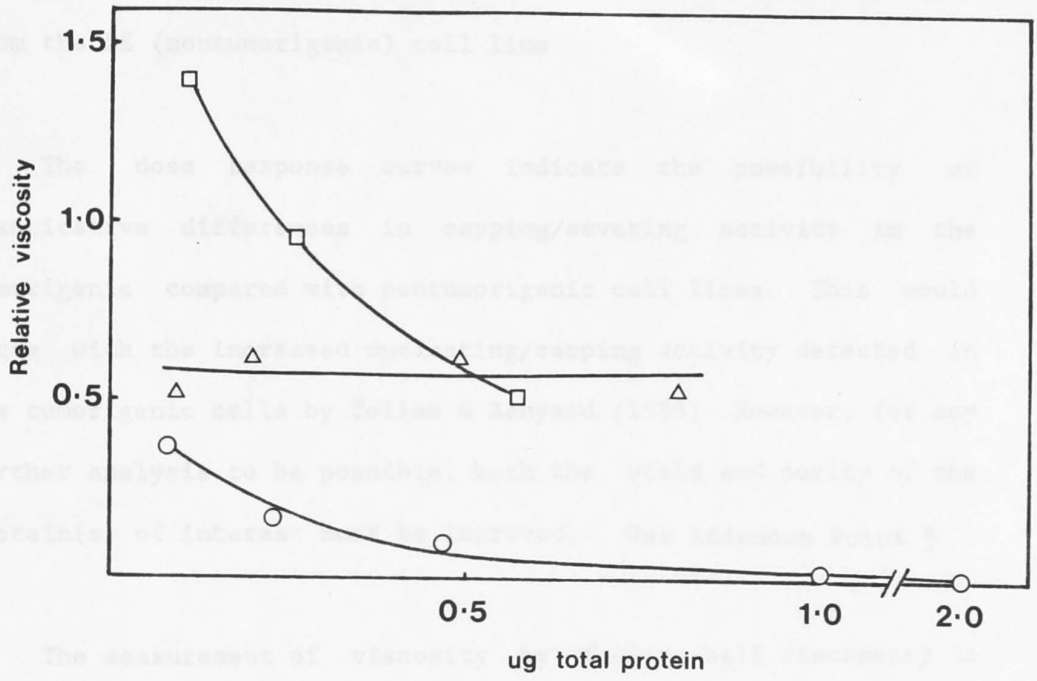
The difference in specific activity in the 5E and 5L 0.6M NaCl washes is not apparent in these falling-ball viscometry assays. The dose response curves suggests that the activity causing reduced actin viscosity may be present in greater amounts in the 5E compared to the 5L 0.6M NaCl wash, and in the 5L 0.1M NaCl wash and the 5L Gu.HCl eluate compared with the corresponding fractions from 5E cells. To investigate the reproducibility of these data, the dose response curves for the Gu.HCl eluates were repeated. The second pair of curves, while differing somewhat from those shown in figure 21, still suggested the Gu.HCl eluate of the 5L (tumorigenic) cells contained a greater concentration of

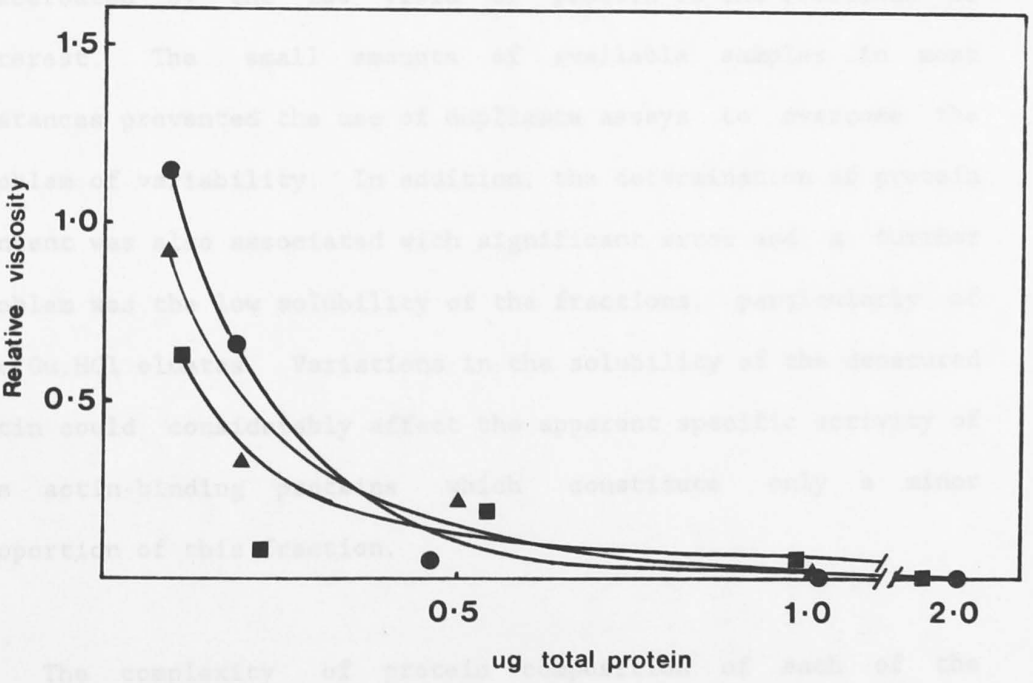
FIGURE 21: Dose-response curves for the reduction of F-actin viscosity by DNase I-affinity chromatography fractions.

Viscosity was assessed by falling-ball viscometry, as described in section 4.2.4, using 80ug G-actin and up to 2ug sample protein in a final volume of 400uL. Viscosity measurements were made 30min after the induction of actin polymerization. The values plotted are viscosity measurements relative to those of actin controls. The protein content of the samples was determined by the Micro-Bradford method. The fractions were from the 5E (nontumorigenic) cell line, upper figure and open symbols, and the 5L (tumorigenic) cell line, lower figure and filled symbols. The identities of the fractions are: 0.1M NaCl wash (□ and ■), 0.6M NaCl wash (○ and ●) and Gu.HCl eluate (△ and ▲).

Note

Values of relative viscosity greater than 1 might be expected to indicate a bundling or crosslinking activity but the effects of such activities are only seen when the concentration of actin-binding protein is close to stoichiometric with the concentration of actin. The concentrations of actin-binding proteins used in these experiments are considerably less than the concentration of actin. Hence the relative viscosities that are greater than 1 are more likely to be an artefact of experimental conditions.


1·5
1·0
0·5
Relative viscosity
0·5
1·0
2·0
ug total protein


1·5
1·0
0·5
Relative viscosity
0·5
1·0
2·0
ug total protein

activity causing a reduced actin viscosity than the Gu.HCl eluate from the 5E (nontumorigenic) cell line.

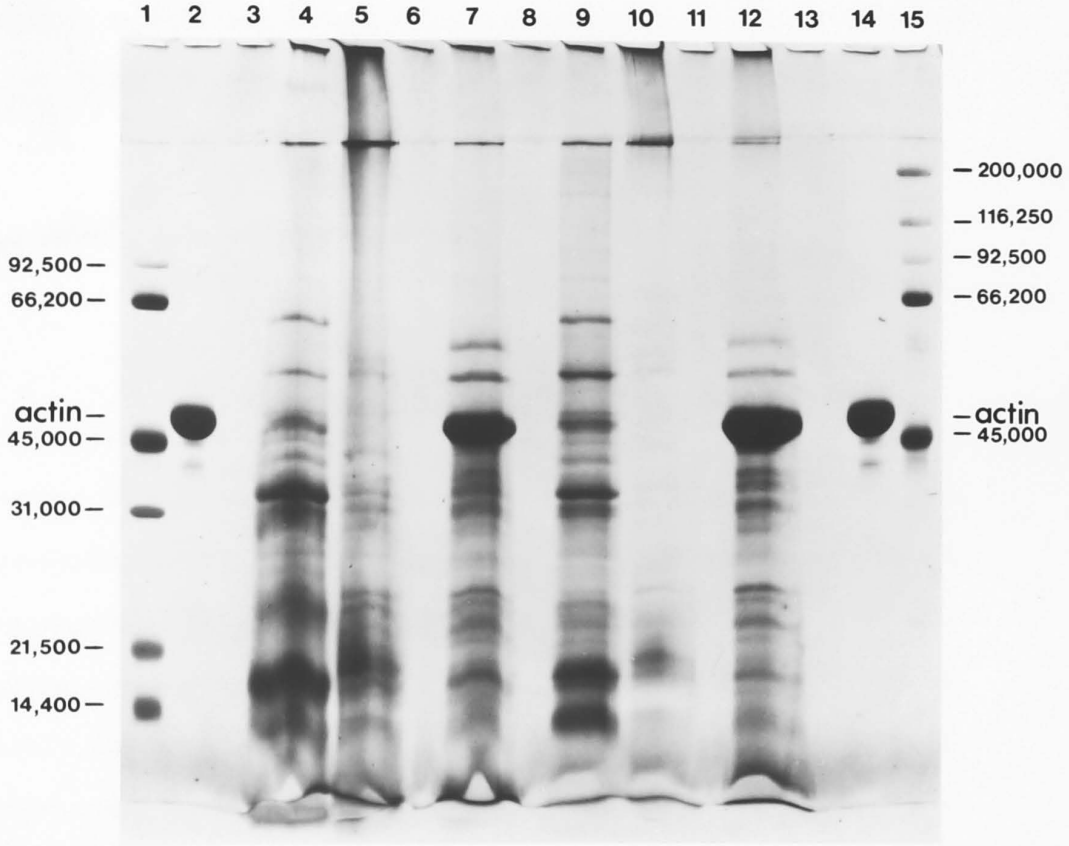
The dose response curves indicate the possibility of quantitative differences in capping/severing activity in the tumorigenic compared with nontumorigenic cell lines. This would agree with the increased nucleating/capping activity detected in the tumorigenic cells by Tellam & Banyard (1985). However, for any further analysis to be possible, both the yield and purity of the protein(s) of interest must be improved. See Addendum Point 3

The measurement of viscosity by falling-ball viscometry is associated with a significant degree of variability and this was exacerbated by the low yield of protein in the fractions of interest. The small amounts of available samples in most instances prevented the use of duplicate assays to overcome the problem of variability. In addition, the determination of protein content was also associated with significant error and a further problem was the low solubility of the fractions, particularly of the Gu.HCl eluates. Variations in the solubility of the denatured actin could considerably affect the apparent specific activity of the actin-binding proteins which constitute only a minor proportion of this fraction.

The complexity of protein composition of each of the fractions is emphasized by the gel shown in figure 22. Whereas 6ug

FIGURE 22: Analysis by SDS-PAGE of DNase I-affinity chromatography fractions from the 5L (tumorigenic) and 5E (nontumorigenic) HeLa/fibroblast hybrid cell lines.

DNase I-affinity chromatography was carried out as described in section 4.2.3. The lyophilized fractions were solubilized in distilled water and the protein content determined by the Micro-Bradford method. Molecular weight standards were applied to the 12% acrylamide gel in lanes 1&15 and rabbit skeletal muscle actin in lanes 2&14. Samples were applied as follows: Lane 4, 5L 0.1M NaCl wash, 20ug protein; Lane 5, 5L 0.6M NaCl wash, 27ug protein; Lane 7, 5L Gu.HCl eluate, 30ug protein; Lane 9, 5E 0.1M NaCl wash, >9ug protein; Lane 10, 5E 0.6M NaCl wash, 7ug protein; Lane 12, 5E Gu.HCl eluate, >6ug protein.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
92,500
66,200
actin
45,000
31,000
21,500
14,400
200,000
116,250
92,500
66,200
actin
45,000

total protein was loaded per lane for the gel shown in figure 19b, the gel shown in figure 22 used loadings of up to 30ug total protein per lane thus allowing visualization of the minor components. For a detailed characterization of the capping/severing activities present in these fractions it is necessary to at least reduce this complexity.

4.3.3 Reversed-Phase HPLC

The low yield of protein from the DNase I-Sepharose column presents a considerable problem for further purification. Reversed-phase HPLC (RP-HPLC) is an attractive method of fractionation of the affinity-purified material because the proteins are mostly low molecular weight and therefore should be suitable for separation by this method. In addition, RP-HPLC is carried out using volatile buffers which can be subsequently evaporated away to concentrate proteins of interest. In developing the separation method, the solubility of the affinity-purified material in 30% acetonitrile was tested. Most of the material was found to be insoluble and was separated as a pellet by centrifugation. The soluble material in the supernatant was, after lyophilization to remove acetonitrile, found to retain the ability to reduce the viscosity of F-actin solutions, as

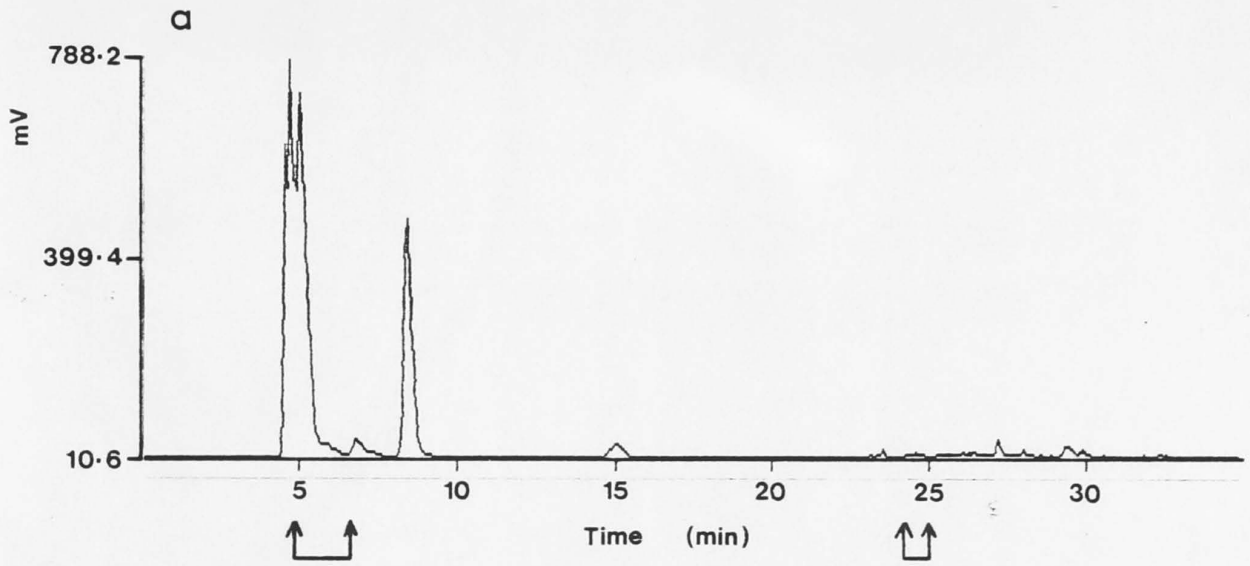
determined by falling-ball viscometry. This led to the development of the method described in section 4.2.9. That is, cell lysates were first fractionated by solubility in 30% acetonitrile. Insoluble material was discarded and the supernatant lyophilized. The lyophilized material was resuspended in water/TFA, insoluble material again pelleted by centrifugation, and the supernatant separated by RP-HPLC.

Figure 23 shows the elution profiles (at 280nm) for single injections of cell extracts from IA3 cn 2.1 (nontumorigenic) and IA3 cn TG (tumorigenic) cells. The elution profiles are significantly different at 5 to 10min after sample injection. Several injections were made with 1min fractions being collected and pooled. The pooled fractions were then lyophilized to remove acetonitrile and TFA, and solubilized in 250mM NaCl buffer for assays of actin-binding activity by falling-ball viscometry. Considerable reduction in viscosity was caused by fractions eluted from the column (as indicated on the elution profiles) at approximately 5 and 24min.

To investigate this activity, further material from the IA3 cn TG cell line was fractionated by the same method and the pooled fractions analysed by SDS-PAGE (Fig. 24). While this gel has considerable artefactual staining, with marked staining between lanes, it is apparent that the activity eluting at 5min that causes a reduction in actin viscosity (lanes 3&5) is not due to

FIGURE 23: The separation by reversed-phase HPLC of extracts from (a) IA3 cn 2.1 (nontumorigenic) and (b) IA3 cn TG (tumorigenic) cells.

The details of the HPLC method are given in section 4.2.9. The elution profiles shown indicate absorbance of the column eluate at 280nm. 1min fractions were collected, lyophilized and assayed by falling-ball viscometry (as described in section 4.2.4) for the ability to reduce the viscosity of F-actin solutions. As indicated by the arrows, fractions eluted at approximately 5min and 24min after sample injection, caused a reduction in the viscosity of F-actin solutions. However, as shown in figure 24, no detectable protein is eluted until 24min after sample injection.


a
788·2
mV
399·4
10·6
5
10
15
20
25
30
Time (min)

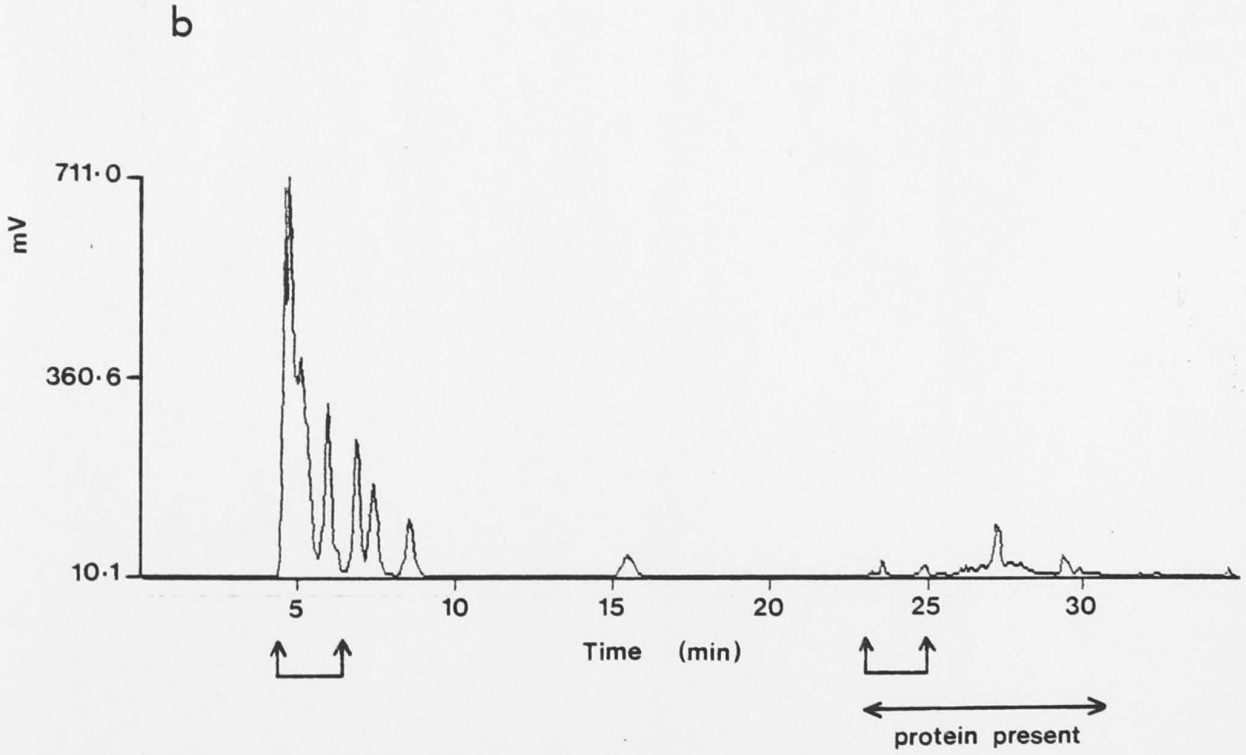
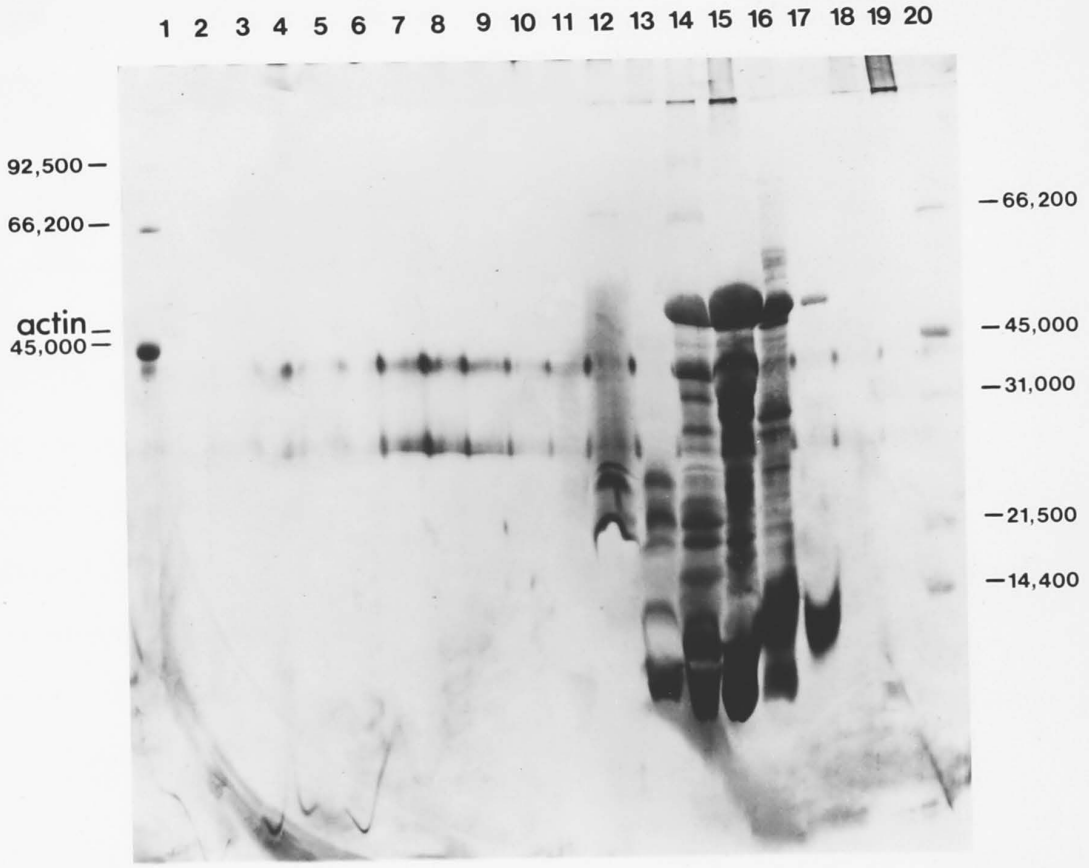

b
711·0
mV
360·6
10·1
5
10
15
20
25
30
Time (min)
protein present

FIGURE 24: Analysis by SDS-PAGE of the protein content of the fractions obtained during RP-HPLC separation of 1A3 cn TG cell extract.

Molecular weight standards were applied to the 15% acrylamide gel in lanes 1&20. Rabbit skeletal muscle actin and a tropomyosin preparation were applied to lanes 2&19, respectively, but loadings were insufficient for detection. The RP-HPLC fractions applied were as follows: Lane 3, 4-5min; Lane 5, 5-6min; Lane 6, 6-7min; Lane 7, 7-8min; Lane 8, 8-9min; Lane 9, 9-10min; Lane 10, 15-16min; Lane 11, 23-24min; Lane 12, 24-25min; Lane 13, 25-26min; Lane 14, 26-27min; Lane 15, 27-28min; Lane 16, 28-29min; Lane 17, 29-30min.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
92,500
66,200
actin
45,000
66,200
45,000
31,000
21,500
14,400

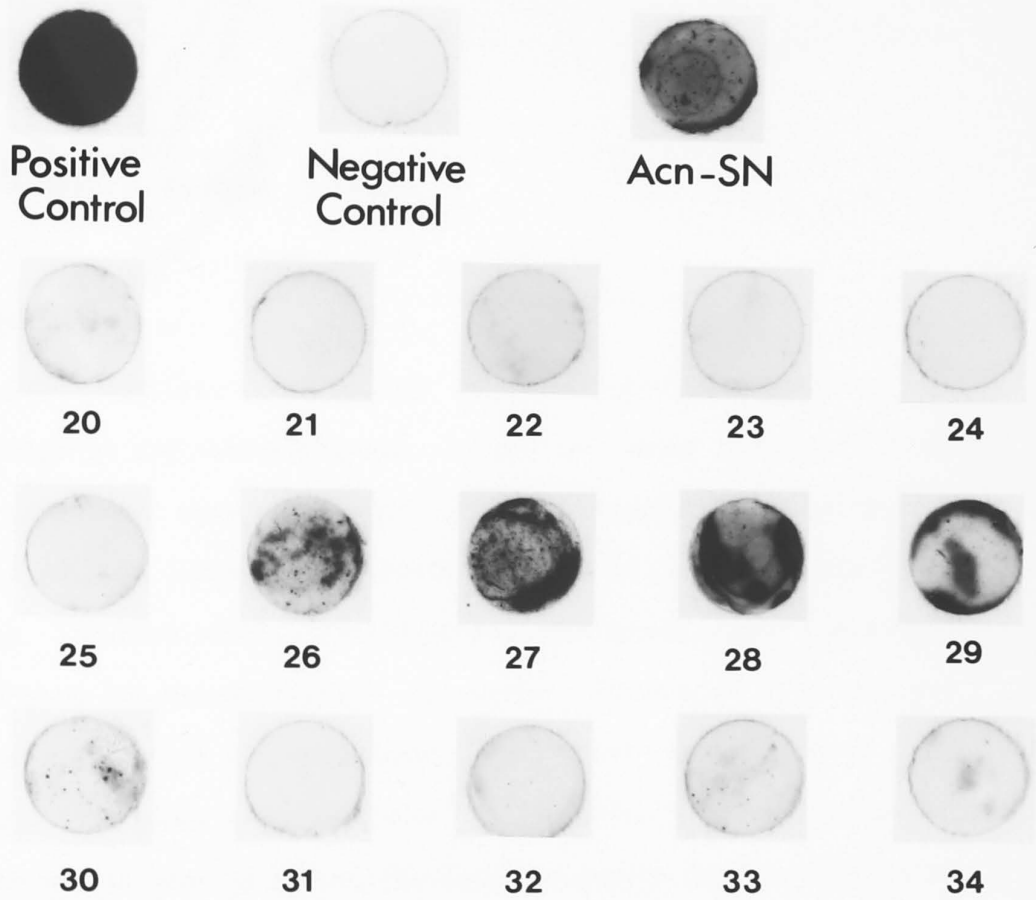
protein. There is however protein in fractions 24&25 (lanes 11&12) which also caused a reduction in the viscosity of actin. Fractions 5&6 were found to have a very low pH (<5) suggesting the effect on actin viscosity may be nonspecific due to the effect of the low pH.

The RP-HPLC fractions which contained protein were analysed for gelsolin content using the dot blot method described in section 4.2.7. That assay showed that (Fig. 25) gelsolin or cross-reactive proteins are present in the cell extract injected onto the HPLC column (ACN-SN) and in fractions 26 to 30, eluted between 25 and 30min. However, the activity detected by falling-ball viscometry was eluted between 23 and 25min and is therefore not due to gelsolin. The failure to detect activity when gelsolin is present suggests that gelsolin is inactivated under these conditions, probably by denaturation.

The separation method was subsequently adjusted to improve the separation of the proteins eluting at 23 to 25mins. Falling-ball viscometry was used to detect the fraction in which the actin-binding protein activity was eluted. This was found to correspond to a minor peak of absorbance at 280nm indicating yield of the active material would be very low. The method is therefore of dubious value for the purification of actin-binding proteins. However, the nonproteinaceous material eluted between 4 and 10min (Fig. 23) may be of interest if it can be identified, since there

FIGURE 25: Analysis of the gelsolin content of IA3 cn TG RP-HPLC fractions by blotting using an anti-gelsolin antibody, as described in section 4.2.7.

The positive control is a lysate from a fibroblast cell line, A164, known to contain gelsolin and the negative control was RP-HPLC column eluate collected after the injection of buffer. The samples tested were the material injected onto the column (Acn-SN) and fractions 20 to 34 eluting between 19 and 34min after injection of the sample.


Positive Control
Negative Control
Acn-SN
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34

is a significant difference in the elution profile of IA3 cn 2.1 and IA3 cn TG cells in this area. However, all that is known of this material is that it absorbs strongly at 280 and 215 nm and that it has a very low pH.

4.3.4 F-Actin-Binding Proteins

To complete the study of actin-binding proteins in tumorigenic and nontumorigenic cells, an assay (section 4.2.10) was developed that would enable the comparison of F-actin-binding proteins. To increase the sensitivity of the assay, cells were first labelled with [^{35}S]methionine, then lysed under conditions favouring the monomerization of actin. The nuclei were pelleted by centrifugation and the lysate supernatant divided into four aliquots for four different sets of conditions. Polymerization of actin was induced in two of the lysate aliquots by the addition of 2mM Mg^{2+} and 0.5mM ATP with one lysate containing calcium and one lacking calcium. Thirty minutes after the induction of polymerization all aliquots were centrifuged (200,000xg, 60min) to sediment F-actin. The supernatant was set aside for analysis while the pellet was resuspended in buffer containing 0.1M NaCl to wash away non-specifically bound proteins and the F-actin was pelleted again by centrifugation. The supernatant was discarded and the

pellet analysed by SDS-PAGE and autoradiography. Figure 26a shows the analysis of the supernatants and figure 26b the pellets. Several different exposure times were used for autoradiography and the figures are composites constructed by selecting the exposure time best suited to visualize the proteins present in each lane.

The protein composition of the pellets differs considerably from the supernatants indicating that the pellets are selectively enriched in a particular fraction of cellular protein. Actin is present in both the supernatants and the pellets. A significant proportion of the cellular actin would be expected to be present in the lysate in a monomeric state, even under conditions favouring actin polymerization because of the presence of actin monomer-sequestering proteins and depolymerizing proteins. This actin would remain in the supernatant after centrifugation.

A careful examination of the pellets failed to demonstrate any qualitative differences in protein composition that could be related to the tumorigenic phenotype. Indeed, the protein composition of the fractions from all the cell lines are remarkably similar. However, the protein composition of both the supernatant and pellets is influenced by calcium concentration. Most noticeable is a protein at 80,000 M_r which, in the absence of calcium is present apparently only in the supernatant. In the presence of calcium the amount of this protein in the supernatant decreases while a protein of identical mobility appears in the

FIGURE 26: Analysis of the F-actin pelleting assay by SDS-PAGE and autoradiography.

The assay was carried out as described in section 4.2.10 using four HeLa/fibroblast hybrid cell lines: 5E and IA3 cn 2.1 (nontumorigenic) and 5L and IA3 cn TG (tumorigenic). Samples were analysed by SDS-PAGE, using 12% acrylamide gels, and subsequently by autoradiography. The positions of molecular weight standards and rabbit skeletal muscle actin is indicated and the identification of the samples as well as the exposure times used for autoradiography is shown in the following tables.

FIGURE 26a: **Supernatants**

Lane	Cell line	Mg^{2+}/ATP	Ca^{2+}	Exposure Time (h)
1	5E	+	-	8
2	5E	-	-	5
3	5E	+	+	8
4	5E	-	+	8
5	5L	+	-	8
6	5L	-	-	8
7	5L	+	+	8
8	5L	-	+	8
9	IA3cn2.1	+	-	8
10	IA3cn2.1	-	-	8
11	IA3cn2.1	+	+	15
12	IA3cn2.1	-	+	15
13	IA3cnTG	+	-	15
14	IA3cnTG	-	-	8
15	IA3cnTG	+	+	15
16	IA3cnTG	-	+	15

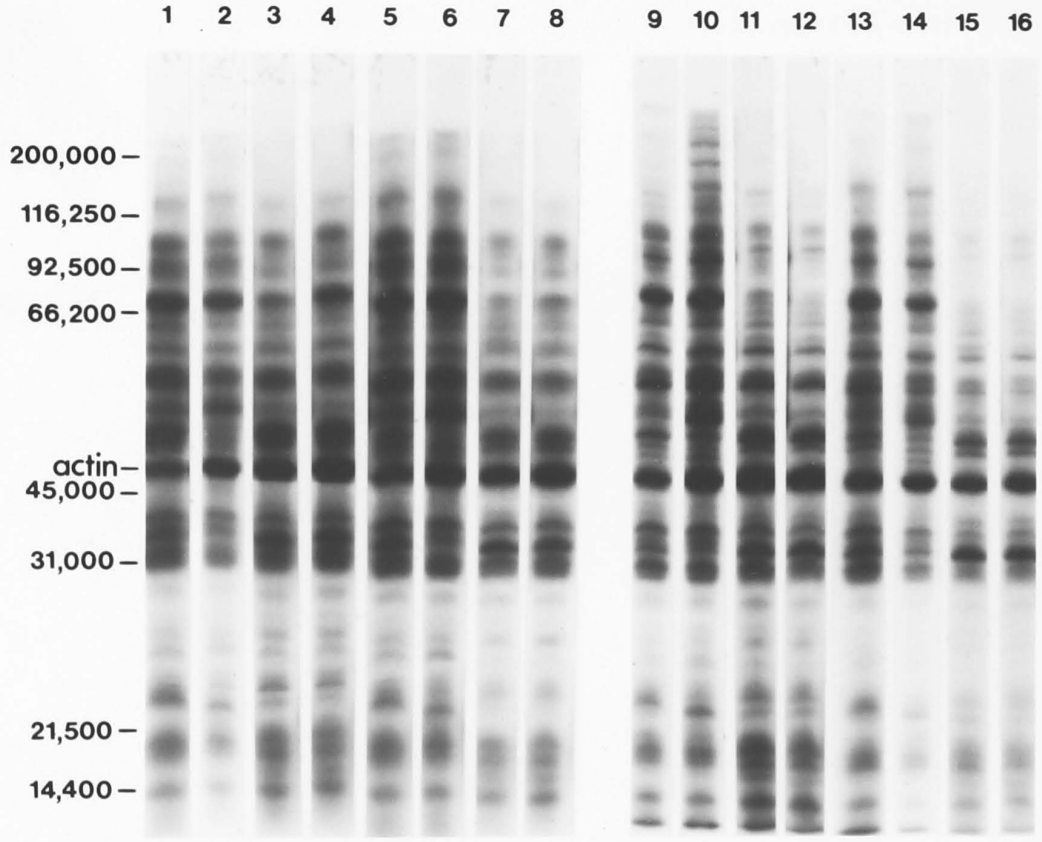
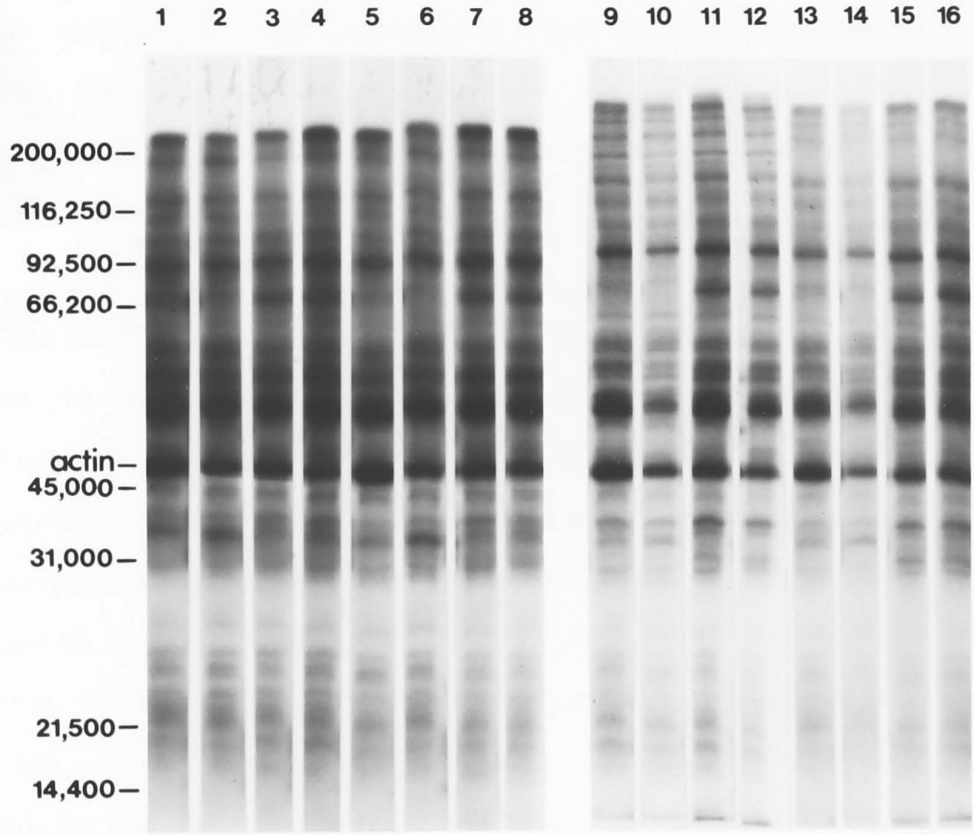

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
200,000
116,250
92,500
66,200
actin
45,000
31,000
21,500
14,400

FIGURE 26b: Pellets

Lane	Cell line	Mg^{2+}/ATP	Ca^{2+}	Exposure time
1	5E	+	-	8
2	5E	-	-	8
3	5E	+	+	8
4	5E	-	+	8
5	5L	+	-	8
6	5L	-	-	8
7	5L	+	+	8
8	5L	-	+	8
9	IA3cn2.1	+	-	8
10	IA3cn2.1	-	-	8
11	IA3cn2.1	+	+	8
12	IA3cn2.1	-	+	8
13	IA3cnTG	+	-	15
14	IA3cnTG	-	-	15
15	IA3cnTG	+	+	15
16	IA3cnTG	-	+	15


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
200,000
116,250
92,500
66,200
actin
45,000
31,000
21,500
14,400

pellet. This is a major protein and its binding to F-actin in the presence of calcium could have a significant effect on actin organization. Another protein of 46,500 M_r is present in the pellets both in the presence and absence of calcium but this protein appears in the supernatant only in the absence of calcium. Thus it would appear that the HeLa/fibroblast hybrid cells contain at least one and possibly two calcium-sensitive F-actin-binding proteins.

Densitometry was used to quantitate the amount of actin present in each fraction. The actin content of the pellet was then expressed as a percentage of the total actin (supernatant plus corresponding pellet). This adjusts for any differences in radioactivity of the four aliquots used to produce the fractions. The results of the quantitation are shown in table 20. The data show that the induction of actin polymerization by the addition of Mg^{2+} and ATP, increases the amount of actin in the pellet, relative to the amount of actin in the corresponding supernatant. The effect of polymerization is greater in the nontumorigenic cells than the tumorigenic cells both in the presence and absence of calcium. To establish the reproducibility of these results, the experiment should be repeated and extended to additional cell lines. Until such verification has been made, conclusions should be made with caution, but if tumorigenic cells have a greater content of capping/severing activity, the degree of polymerization of actin in these cells would be expected to be less than in the

TABLE 20: The distribution of actin between supernatant and pellet during the F-actin pelleting assay, as determined by densitometry of the autoradiograms.

Cell line	Mg^{2+}/ATP	Ca^{2+}	% actin in pellet
5E	+	-	71
5L	+	-	66
IA3cn2.1	+	-	64
IA3cnTG	+	-	45
5E	-	-	10
5L	-	-	38
IA3cn2.1	-	-	10
IA3cnTG	-	-	1*
5E	+	+	37
5L	+	+	44
IA3cn2.1	+	+	34
IA3cnTG	+	+	37
5E	-	+	10
5L	-	+	34
IA3cn2.1	-	+	14
IA3cnTG	-	+	32

* value doubtful; sample may not have been completely solubilized.

nontumorigenic cells thus resulting in the different amounts of actin in the pellets. The effect of calcium concentration on the degree of polymerization would indicate that a calcium-sensitive activity is of some importance to actin organization in these cells.

4.4 Conclusions

The experiments described in this chapter demonstrated the protein composition of the Triton-insoluble pellets, of the actin monomer-binding and of the F-actin-binding pools to be similar in tumorigenic and nontumorigenic HeLa/fibroblast hybrid cells. This suggests that there is no change in the expression of actin-binding proteins associated with the expression of a tumorigenic phenotype. However, tumorigenic cells may contain increased amounts of F-actin capping/severing activities attributable to proteins other than gelsolin. Tellam & Banyard (1986) detected increased amounts of an actin nucleating/capping/severing activity in crude cell lysates from tumorigenic, relative to nontumorigenic, HeLa/fibroblast hybrid cells, but the gelsolin content of these tumorigenic and nontumorigenic cells was found to be similar. However, the amount

See Addendum Point 3

of gelsolin in the HeLa/fibroblast hybrid cells and the parental HeLa cell line (D98 AH2) is less (by a factor of 10) than the amount of gelsolin in primary fibroblasts (Banyard *et al.*, personal communication). This suggests that while gelsolin may be a major actin-binding protein in fibroblasts, the regulatory function of gelsolin may be performed by other capping/severing proteins in cells of epithelial origin. Increased amounts of this type of activity could be important in the disorganization of the microfilaments seen in the tumorigenic cells. Indeed, the microinjection of capping proteins from mammalian brain (63,000 M_r) and from *Physarum polycephalum* (42,000 M_r) into epithelial or fibroblastic cells results in the disruption of microfilament organization and contact sites of actin filaments (Fuchtbauer *et al.*, 1983). More recently, Magargal & Lin (1986) have detected increased levels of an endogenous cytochalasin-like activity in chick embryo fibroblasts transformed by RSV (the cytochalasins are fungal metabolites known to cap the barbed ends of actin filaments).

While the composition of the protein pools examined was found to be similar for the tumorigenic and nontumorigenic cells, it is possible that differences in the actin-binding proteins of tumorigenic and nontumorigenic cells might be induced by mechanisms other than changes in concentration. Minor structural alterations might alter the affinity with which the proteins bind to actin or the sensitivity of the protein to calcium or other

regulatory molecules. The phosphorylation state of a protein might also influence actin-binding properties or the activity of the protein towards actin. Small variations in the relative amounts of actin-binding proteins, not detected by the methods used in this study, could have a significant effect on actin organization. For example, decreased amounts of tropomyosin, a protein which protects F-actin against the action of severing proteins, in combination with increased capping/severing activity in the cell would be expected to result in fragmentation of the actin filaments. These potential differences will be considered further in chapter 5.

Chapter 5

CONCLUSIONS AND FUTURE PROSPECTS.

5.1 The Organization of the Microfilament System in Malignant Cells

The re-expression of tumorigenicity in the HeLa/fibroblast somatic cell hybrid system is associated with a loss of microfilament organization. This loss of organization is readily apparent as a reduction in the number, diameter and length of stress fibres and, in addition, some of the tumorigenic cells contain structures which must be aggregates of F-actin. While these aggregates are not present in all tumorigenic cells they were never observed in nontumorigenic cells and may therefore, represent an abnormal actin structure unique to tumorigenic cells.

A disorganization of microfilament structure is typical of transformed cells of mesenchymal origin, including fibroblasts, but further study will be necessary to clarify whether alterations in microfilament organization are generally associated with the malignant transformation of other cell types. The organization of microfilaments in epithelial cells, for example, is complex and dependent on cell-cell and cell-substratum interactions (Ben-Ze'ev & Amsterdam, 1986) as well as on the agent causing the

transformation (Keski-Oja *et al.*, 1983). It has been suggested that the disorganization of microfilament structure is correlated with metastatic potential rather than tumorigenicity (Zachary *et al.*, 1986). With the HeLa/fibroblast hybrid cell system it is not possible to assess metastatic potential. The investigation of any association between metastatic potential and microfilament organization is an area for future study.

It is clear that microfilament organization is altered in association with the re-expression of the tumorigenic phenotype but microscopic examination of the cells with a higher degree of resolution would enable the alterations in microfilament organization to be determined in more detail. For example, it would be of interest to determine whether the disruption of stress fibres is associated with a reduction in mean actin filament length or with a disruption of the interaction between actin filaments and the cell membrane. In addition, knowledge of the organization of actin in the aggregates and the relationship between the F-actin aggregates and the plasma membrane may aid in deducing the mechanism leading to the formation of these aggregates. The resolution of fluorescence microscopy can be increased by using fluorescence enhancement techniques in conjunction with high magnification but for a detailed examination, electron microscopy would be preferable. Appropriate fixation of cells (Maupin & Pollard, 1983; Boyles *et al.*, 1985) will enable the visualization of individual actin filaments while

decoration of actin filaments with myosin subfragment-1 (Begg *et al.*, 1978) not only enhances the visualization of actin filaments but enables the polarity of the filaments to be determined. Immunoelectron microscopy (Chen & Singer, 1982; Langanger *et al.*, 1984) utilizing antibodies against actin-binding proteins may also be useful in determining the relationship between the proteins and microfilament organization.

The simplest technique for characterization of the F-actin aggregates would be immunofluorescence using antibodies against actin-binding proteins which may be expected to be present in the aggregates. Carley *et al.* (1985) have utilized immunofluorescence in this way to study actin aggregates of fibroblasts transformed by several different agents. They found that the actin-binding proteins alpha-actinin, caldesmon and fimbrin but not tropomyosin were present in the aggregates. However, actin structures possibly similar to the F-actin aggregates which are formed in chick embryo fibroblasts transformed by RSV were shown to contain alpha-actinin, myosin and tropomyosin (Boschek *et al.*, 1981). Monoclonal antibodies against some of these actin-binding proteins are now available and would be ideal for further studies using immunofluorescence microscopy.

The microfilaments of live cells can be labelled with NBD-phallacidin (Barak *et al.*, 1981) and, while continuous observation under UV illumination is not possible because of

bleaching of the fluorescence, intermittent observation possibly in combination with continuous phase contrast observation, should provide information on the stability of the aggregates and whether the aggregates are formed as the result of stress fibre disruption. Aggregates of F-actin have been reported to be associated with membrane protrusions (Boschek *et al.*, 1981) and, as indicated above, electron microscopy would confirm whether a similar association is present in the HeLa/fibroblast hybrid cells. Malignant cells in general exhibit increased motile activity at the cell membrane and it is possible that the F-actin aggregates may be associated with vesicles formed at the membrane and are possibly destined to fuse with lysosomes. Observation of the aggregates in combination with a specific marker for lysosomes would test this possibility. If the aggregates prove to be stable structures, or are able to be stabilized in some way, such as by the addition of phalloidin (Carraway & Weiss, 1985), it might be possible to separate the aggregates by centrifugation on sucrose gradients thereby allowing analysis of their protein composition by SDS-PAGE, and, given sufficient material, further purification.

5.2 The Actin Content of Malignant Cells

The total actin content of the tumorigenic HeLa/fibroblast hybrid cells, as measured by the DNase I-inhibition assay, is significantly less (by 35%) than the actin content of the nontumorigenic cells but further studies will be necessary to establish whether such a reduction in actin content is a generalized feature of malignant cells. Leukaemic lymphocytes have been reported to contain significantly less actin than normal lymphocytes (Atkins & Anderson, 1982; Stark *et al.*, 1982) with the magnitude of reduction (36%) being similar to that detected in the HeLa/fibroblast hybrid cells. However, the comparison of nonadherent with adherent cell systems should be made with caution. Those studies that have been made of the actin content using adherent malignant cell lines have produced variable results. Some of this variation is probably attributable to the use of different methods to determine actin content. The DNase I-inhibition assay used in this study to measure actin content is highly sensitive and specific.

The reduction in the total actin content of the tumorigenic HeLa/fibroblast hybrid cells may result from a general reduction

in all actin isoforms or the selective reduction of certain isoform(s), since the DNase I-inhibition assay does not distinguish between actin isoforms. The major actin isoforms can be separated by isoelectric focusing (Stark *et al.*, 1982; Leavitt *et al.*, 1985; Otey *et al.*, 1986). However, while the pattern of actin isoforms is known to vary with cell type and differentiation state (Otey *et al.*, 1986; Strauch *et al.*, 1986), the significance of these differences in isoform content, with regard to the organization of microfilaments, is not clear since no differences in isoform function or cellular location have yet been conclusively demonstrated. The different actin isoforms display greater than 90% sequence homology with variability mostly restricted to a region near the amino terminus (Otey *et al.*, 1986). If this region of the molecule is a binding site for actin-binding proteins, then it is possible that differences in isoform ratios could indirectly influence actin organization. Altered ratios of actin isoforms and the synthesis of a mutant actin have been detected in transformed fibroblasts (Witt *et al.*, 1983; Leavitt *et al.*, 1985). Future research into the structure and properties of the actin molecule, the mechanisms of actin polymerization and interaction with regulatory proteins will be of considerable importance in determining the significance of these apparently minor structural differences in the actin molecule.

Actin synthesis, relative to total protein synthesis, is not decreased in the tumorigenic HeLa/fibroblast hybrid cells

indicating that a specific suppression of actin synthesis is not the cause of the reduced total actin content of the tumorigenic cells. Determination of actin mRNA concentration in the tumorigenic and nontumorigenic HeLa/fibroblast hybrid cells would confirm that actin sythesis is not suppressed in the tumorigenic cells and it would also be of interest to determine whether there is coordinate regulation of actin and actin-binding protein gene expression. Despite the reduction in total actin content of tumorigenic cells, the pool of actin monomer is in all cells maintained at about 35% of the total actin content. This suggests that it is important for normal cellular functioning to maintain this balance between actin polymer and monomer. Most of the actin monomer pool probably consists of actin complexed to profilin and the coordinate regulation of profilin and actin synthesis could be one mechanism by which the appropriate ratio of profilin to actin might be maintained.

The synthesis of alpha- and beta-tubulin is regulated by tubulin concentrations in a negative feedback mechanism through non-transcriptional control of mRNA levels (Cleveland & Havercroft, 1983) and it is possible that the regulation of actin synthesis occurs by a similar mechanism. Since the total actin content of the tumorigenic cells is reduced while the actin monomer pool is maintained at a constant proportion of the total actin content, then the actin monomer concentration must be less in the tumorigenic cells. If the actin monomer regulates levels

of actin mRNA by negative feedback, then the concentration of actin mRNA, and consequently actin synthesis, might therefore be expected to be greater in the tumorigenic cells. Distinction between the mRNA's of the different actin isoforms (Leof *et al.*, 1986; Strauch *et al.*, 1986) would also be informative in view of the suggestion that the relative abundance of actin isoforms may be altered in tumorigenic cells.

The concentration of any protein at steady state results from the balance between the rates of synthesis and degradation. Actin synthesis is not suppressed in the tumorigenic HeLa/fibroblast hybrid cells and, therefore, the reduced total actin content in the tumorigenic cells must result from an increased rate of actin degradation. The half-life of actin is greater than 3 days in growing 3T3 cells (Fine & Taylor, 1976) while the cell cycle of the HeLa/fibroblast hybrid cell is approximately 24 hours. This makes it difficult to determine the rate of actin degradation by following the loss of radioactive label since the diluting effect of cell division causes the specific radioactivity to decrease faster than does actin degradation. However, it should be possible to measure the rate of actin degradation in the HeLa/fibroblast hybrid cells using dual label techniques (Clark & Zak, 1981). Such measurements would confirm whether the rate of actin degradation is indeed increased in the tumorigenic cells and whether the increased rate of degradation is restricted to actin or is part of a generalized increase in the rate of turnover of

all cellular proteins. It is possible that actin degradation may occur in conjunction with the degradation of actin-binding proteins: the rate of gelsolin degradation has been found to be increased in the tumorigenic HeLa/fibroblast hybrid cells (Banyard *et al.*, personal communication). The coordinate degradation of actin and actin-binding proteins would help to maintain the appropriate ratios of actin to actin-binding proteins thereby ensuring that the appropriate balance of actin monomer to actin polymer is maintained. It was also suggested earlier that the F-actin aggregates may be enclosed in vesicles and subsequently degraded in lysosomes. This would result in the coordinate degradation of actin and associated actin-binding proteins.

Actin monomer is more susceptible to proteolysis than is actin polymer and it was therefore suggested that the presumptive increase in actin degradation results from an increased turnover of actin polymer with actin monomer thereby increasing the availability of actin monomer for degradation. The increased turnover of actin monomer with actin polymer was, in turn, suggested to result from the instability of polymeric actin structures in the tumorigenic cells. The increased rate of actin degradation, and the reduced actin content of the tumorigenic cells, would therefore be a consequence and not the cause of the disorganization of the microfilament system in the tumorigenic cells. To investigate this hypothesis it would be useful to compare the rates of turnover of actin monomer with actin polymer

and the stability of polymeric actin structures in the tumorigenic and nontumorigenic HeLa/fibroblast hybrid cells. As discussed earlier, the microfilaments of live cells can be labelled with NBD-phallacidin and studied for several hours with intermittent examination by UV illumination. Such studies would allow the qualitative comparison of the stability of polymeric actin structures. However, phallicidin, through its binding to F-actin, does cause some stabilization of polymeric structures. Rhodamine- or fluorescein-labelled actin retain normal polymerization properties and the incorporation of these fluorescently labelled actins into polymeric actin is often associated with an increase in fluorescence (Tait & Frieden, 1982a&b). The microinjection of these labelled actins into cells should allow, using computer aided imaging and fluorescent photobleach recovery techniques, determination of the rate of turnover of actin polymer with actin monomer.

5.3 The Regulation of Microfilament Organization

If the increased rate of actin degradation and reduced total actin content of the tumorigenic cells is the consequence of the instability of polymeric actin structures in these cells, then the

critical alteration in the tumorigenic cells is the loss of the ability to form a highly organized, stable microfilament system. The organization of actin is controlled at all levels by a diversity of actin-binding proteins (Pollard & Cooper, 1986). The actin-binding proteins of the HeLa/fibroblast hybrid cells were compared using different techniques to select for particular pools of cytoskeletal proteins. These pools were a Triton-insoluble pellet enriched for actin and actin-binding proteins, cell extracts enriched for actin monomer-binding proteins by DNase I-affinity chromatography, and F-actin-binding proteins sedimented in association with F-actin. The qualitative comparison between the tumorigenic and nontumorigenic cells for each of these three pools did not detect any differences in protein composition associated with the expression of tumorigenicity.

The Triton-insoluble and F-actin pellets both exhibited a relatively complex protein composition making the comparison of minor protein components difficult but implicating the involvement of a large number of proteins in the control of microfilament organization. The organization of actin is finely balanced and consequently it is sensitive to changes in the concentration of even minor protein components. The protein composition of the DNase I-affinity chromatography fractions was less complex than the Triton-insoluble pellet but the yield of protein was low thus reducing the sensitivity of detection. While it remains likely that differences in the actin-binding proteins of tumorigenic and

nontumorigenic HeLa/fibroblast hybrid cells exist, to detect such differences it will be necessary to increase the yield and purity of the fractions of interest and to use techniques which will increase the sensitivity and/or which will detect differences in properties rather than quantities of the actin-binding proteins.

5.3.1 Purification of Actin-Binding Proteins by DNase I-Affinity Chromatography

A major problem with the use of DNase I-affinity chromatography to isolate actin-binding proteins from the HeLa/fibroblast hybrid cells was the low yield of proteins of interest. To increase the yield it will be necessary to either grow increased numbers of cells or to isolate the proteins of interest by alternative techniques. The growth of the HeLa/fibroblast hybrid cells to large numbers in tissue culture flasks is expensive and cumbersome. Increased numbers of cells were grown on Cytodex 3 microcarriers (section 4.2.8) but this technique was not considered to provide a significant increase in cost efficiency. It is of interest to note that in published reports of the purification of actin-binding proteins using DNase I-affinity chromatography, the cells used are generally from a source that provides large numbers at little cost. Organisms such

as *Acanthamoeba* and *Physarum* are readily grown in large quantities for low cost while vertebrate sources such as bovine or porcine brain, liver, kidney etc. are similarly available cheaply and in quantity.

A useful approach would be to isolate individual actin-binding proteins from an alternative easily available source such as vertebrate brain, kidney or smooth muscle, or human tumour tissue if it is available, and then to characterize the proteins isolated and raise polyclonal or monoclonal antibodies against proteins of interest. The antibodies could then be used to (1) identify and localize immunologically cross-reactive proteins in the HeLa/fibroblast hybrid cells, (2) isolate these proteins from the hybrid cells by immunoaffinity chromatography techniques, and (3) to quantitate, by immunoprecipitation or ELISA techniques, the amounts of the proteins in the tumorigenic and nontumorigenic cells. Such techniques would be considerably more sensitive than the qualitative comparison provided by SDS-PAGE subsequent to purification.

DNase I-affinity chromatography alone does not provide a sufficient degree of purity for the detailed characterization of actin-binding proteins. Additional methods, such as ion exchange chromatography or gel filtration, will be necessary to further separate the proteins in the DNase I-affinity chromatography fractions. For these techniques to be used the protein yield in

the DNase I-affinity chromatography fractions must first be considerably increased.

Once an increased yield and purity of proteins of interest has been achieved, the techniques of falling-ball viscometry and pyrene-actin fluorescence enhancement can be used to characterize actin-binding protein activities in some detail. The availability of highly pure preparations would also allow data on amino acid composition, peptide maps and possibly some sequence data to be obtained. Such data would be invaluable in the comparison of the proteins with known actin-binding proteins and in the detection of abnormal proteins.

An increased amount of a nucleating and capping activity has previously been detected in crude lysates from the tumorigenic in comparison with the nontumorigenic HeLa/fibroblast hybrid cells (Tellam & Banyard, 1986) and an increased amount of F-actin capping activity has been detected in chick embryo fibroblasts transformed by RSV (Magargal & Lin, 1986). In addition, the microinjection of capping proteins into vertebrate fibroblasts induces the disruption of microfilament structure (Fuchtbauer *et al.*, 1983). These results suggest that an increase in actin

capping/severing activity may be of some importance in the loss of microfilament organization in malignant cells and consequently it is important that this activity in the tumorigenic and nontumorigenic HeLa/fibroblast hybrid cells is further characterized. As suggested earlier, appropriate antibodies would enable quantitation, by immunoprecipitation or ELISA techniques, of specific actin filament capping and severing proteins, but any differences in activity may not be due to differences in concentration of the protein. Hence, it will be important to quantitate the specific activity of these purified proteins in tumorigenic and nontumorigenic cells to differentiate between differences in quantity or intrinsic activity of the proteins. In the latter case, structural studies would be required to identify the cause of the differences in intrinsic activity of the proteins from tumorigenic and nontumorigenic cells.

5.3.2 The Regulation of Actin-Binding Protein Activity

There are a number of mechanisms whereby the activities of actin-binding proteins might be regulated. It is important to consider these mechanisms and ways in which alterations of actin-binding protein activity could occur in association with malignant transformation.

Firstly, actin-binding protein activity can be regulated by control of intracellular concentration. In addition, if an actin-binding protein exists as multiple isoforms, with each isoform exhibiting differences in structure and/or properties, then changes in the relative abundance of isoforms might significantly affect the net actin-binding protein activity without altering the total intracellular concentration of the actin-binding protein. There is some evidence for this mechanism. For example, tropomyosin is known to be present in individual cells as multiple isoforms distinguished by molecular weight variants and in their affinities of binding to F-actin (Keiser & Wegner, 1985). Alpha-actinin of platelets has also been demonstrated to exist as distinct isoforms differing in the Ca^{2+}-sensitivity of their F-actin-binding activity and in the efficiency with which actin is cross-linked (Landon *et al.*, 1985). The expression of isoforms may depend on cell type and differentiation state. The most extreme example of tissue-specific expression comes from the actin-binding proteins of muscle cells. The muscle form of alpha-actinin is only found in muscle cells and, unlike nonmuscle alpha-actinin, the activity of muscle alpha-actinin is calcium sensitive.

The studies described in chapter 4 did not detect any changes in the absolute concentration of actin-binding proteins associated with the expression of a tumorigenic phenotype but, as discussed

previously, more sensitive techniques might detect minor changes in concentration. However, the relative abundance of isoforms of an actin-binding protein might be altered in malignant cells, without altering the total concentration of the protein, through changes in gene expression or isoform-specific changes in the rate of protein turnover. The balance of tropomyosin isoforms has been reported to be altered in certain transformed cells with the higher molecular weight isoforms, which bind to F-actin with greater affinity, tending to be lost. The binding of tropomyosin to F-actin protects the filaments against severing proteins and the loss of the high affinity forms could, therefore, increase the susceptibility of actin filaments to severing activity

A second mechanism by which actin-binding proteins might be regulated is through structural modification, of either a covalent or a non-covalent nature. Covalent modification may be caused by phosphorylation, reversed only by the action of specific phosphatases, while non-covalent modification may result from the binding of Ca^{2+}, lipids or other intracellular molecules and, possibly, sensitivity to intracellular pH. Both covalent and non-covalent modifications, through the induction of conformational changes in the actin-binding protein, could result in alterations in intrinsic activity.

Many of the actin-binding proteins are phosphoproteins and in some instances phosphorylation has been shown to alter the

activity of the actin-binding protein. For example, phosphorylation of MAP-2 reduces the affinity with which it binds to and cross-links actin (Akiyama *et al.*, 1986; Sattilaro, 1986) while phosphorylation of erythrocyte ankyrin reduces the affinity with which spectrin binds to ankyrin (Lu *et al.*, 1985) thereby influencing attachment of the microfilaments to the erythrocyte membrane. A more complex effect is exhibited by an F-actin capping protein from *Physarum polycephalum*, cap 42(a+b), which requires calcium for activity only when the cap 42(b) subunit is phosphorylated (Maruta & Isenberg, 1984). Phosphorylation is probably important in the process of malignant transformation since many of the transforming proteins of the acutely transforming retroviruses possess tyrosine-specific protein kinase activity and the postulated receptor of the phorbol ester tumour promoters is protein kinase C. The v-*fgr* oncogene of the Rasheed-Gardner feline sarcoma virus is a hybrid of an actin and a tyrosine protein kinase gene suggesting that actin-binding proteins may be important targets for phosphorylation associated with malignant transformation (Naharro *et al.*, 1984). Vinculin and talin, both proteins postulated to be involved in the attachment of actin filaments to the plasma membrane, are possible targets for phosphorylation during malignant transformation. Vinculin is phosphorylated by the tyrosine protein kinase of RSV and protein kinase C, while talin is phosphorylated by protein kinase C. However, the effect of phosphorylation on the activities of these proteins is not known.

A number of capping/severing proteins including gelsolin, villin and fragmin exhibit activity only in the presence of micromolar concentrations of Ca^{2+}. Cross-linking of actin filaments is also calcium-sensitive through the action of caldesmon, a protein which binds to actin and blocks the cross-linking activity of ABP only in the absence of calcium, and through actinogelin whose cross-linking activity is inhibited directly by calcium. An increased intracellular calcium ion concentration acts as a second messenger system for certain extracellular signalling molecules (for example, mitogens) and, if proteins of tumorigenic and nontumorigenic cells differ in sensitivity to calcium, then the intracellular response to calcium fluxes induced by such extracellular signals will also differ.

There is some evidence for the regulation of actin-binding protein activity by actin-binding protein-lipid interactions. For example, profilin binds to actin monomer with high affinity to form a stoichiometric complex, profilactin, which does not polymerize. However, profilin also interacts with anionic phospholipids such as phosphatidylinositol and this interaction reduces the affinity with which profilin binds to actin resulting in the release and subsequent polymerization of actin. In addition, interactions with lipids may be important in the attachment of actin filaments to the plasma membrane. Covalent linkages with phosphatidylinositol have been suggested to be a

mechanism whereby integral membrane proteins are attached to the phospholipid bilayer (Low *et al.*, 1986) and aggregates of actin, actin-binding proteins and lipids have been reported to form (Burn *et al.*, 1985). It is possible that the interaction of certain actin-binding proteins (such as vinculin or talin) with a hydrophobic lipid environment induces a conformation that increases the affinity of the binding interactions leading to the attachment of actin to the plasma membrane. Changes in structure and the rate of turnover of lipids might therefore influence the activities and interactions of actin-binding proteins.

A third mechanism by which the regulation of actin-binding protein activity might occur is through interaction with other molecules. This mechanism is closely related to the second mechanism, discussed above, in that the activity of actin-binding proteins may be influenced through interactions with ions, such as calcium, or with lipids, but in addition interaction with other proteins will be significant. In the complex environment of the cell competing interactions are likely to be common, and the net result will depend on the strength and specificity of the individual interactions. For example, caldesmon binds to F-actin in the absence of calcium and inhibits cross-linking by ABP but in the presence of calcium, caldesmon binds preferentially to calmodulin and is dissociated from F-actin thereby allowing cross-linking to occur. Any structural alteration to a protein that affects its binding properties, or an alteration in the

relative abundance of isoforms with distinct affinities of binding, will affect the ability of an actin-binding protein to compete for binding sites on actin and could, therefore, affect the net activity exhibited.

A fourth factor important in the regulation of actin-binding protein activity, is that of intracellular distribution, with the distribution in turn being dependent on the properties and interactions of the individual actin-binding proteins. For example, the presence of tropomyosin bound to F-actin with high affinity probably restricts the binding of capping and severing proteins, such as gelsolin and fragmin, to the ends of actin filaments and areas of the cell where tropomyosin is absent. As a consequence tropomyosin is found in stable F-actin strutures, such as stress fibres, while capping and severing proteins are generally found in regions of rapid microfilament reorganization. Actin-binding proteins with affinity for membrane lipids or membrane-bound proteins are likely to be distributed between soluble and membrane-bound fractions of the cell. This distribution of proteins between soluble and membrane-bound pools might be influenced not only by the properties of the actin-binding protein but also by the lipids present in the membrane. For example, activation of platelets by thrombin induces the hydrolysis of phosphatidyl inositol 4,5-bisphosphate to diacylglycerol and phosphatidyl trisphosphate. The presence of diacylglycerol in the platelet membrane in turn induces the

formation of complexes of alpha-actinin, actin and lipid (Burn *et al.*, 1985). This altered distribution of actin and at least one associated actin-binding protein may be important to the alterations of microfilament organization and platelet shape that occur when platelets are activated.

In comparing actin-binding protein activities of tumorigenic and nontumorigenic cells it will be important to consider these regulatory mechanisms as potential means by which differences in specific activities might be induced thereby causing the altered microfilament organization that is associated with malignant transformation. Structural changes in the proteins affecting the affinity of binding to actin, lipids, calcium or other molecules could be induced by gene mutations: point mutations in critical binding sites can dramatically alter protein conformation and binding properties. Altered gene expression could result in the presence of an actin-binding protein in an incorrect cell type or at the wrong stage of differentiation. Phosphorylation may also be an important mechanism by which alterations of actin-binding protein activity could be induced. Assessment of these possibilities will require careful characterization of actin-binding proteins of interest.

5.4 Final Conclusions

The organization of the microfilament system is complex and sensitive to many regulatory agents. The vital role of the microfilament system in many normal cellular functions is reflected in the complexity of regulatory mechanisms and the sparsity of pathological conditions found to be due to abnormalities in the microfilaments (most abnormalities are likely to be lethal to the cell). However, the evidence for alterations in microfilament organization associated with malignant transformation is strong.

It seems likely that, because of the complexity of the microfilament system, disruption of normal organization associated with malignant transformation may occur by a variety of mechanisms such as phosphorylation of actin-binding proteins, changes in actin or actin-binding protein isoform ratios or the expression of these isoforms in an inappropriate cell type, and structural changes leading to altered properties or activities.

The study of oncogenes has demonstrated that transformation can be induced through a number of mechanisms. For example, the

pathway by which extracellular signals are transmitted across the plasma membrane can be disrupted at the level of growth factors, growth factor receptors or growth factor receptor protein kinase activity. Similarly it would appear that disruption of the microfilament system can also be achieved by targeting different regions of the regulatory system.

While the evidence suggests that disruption of the microfilament system is an important factor in the acquisition of a tumorigenic phenotype, it is still not clear whether this alteration is a primary alteration leading to malignancy or a secondary alteration that occurs as a consequence of other changes. Harris (1986) suggests that cells may normally continue to multiply until differentiation induces the cessation of growth. Any event that produces a stable heritable block to differentiation would then result in continuous cell multiplication. Therefore, mutated or functionally abnormal oncogenes may act by impeding the process of terminal differentiation in the cell. The organization of microfilaments depends on cell type and differentiated state. The organization of actin into the contractile apparatus, for example is typical of differentiated muscle cells while a membrane skeleton of cross-linked actin filaments is typical of mature discoid erythrocytes and dominant stress fibres are typical of fibroblasts maintained *in vitro*. If malignancy is the result of abnormal differentiation then the organization of the microfilament system

would be expected to reflect the undifferentiated state. However, it is also possible that disruption of microfilament organization may be one mechanism by which the normal differentiation process can be blocked. The microfilaments are involved in many cellular functions and hence a particular organization of microfilaments may be essential to allow the cell to achieve the functions typical of its differentiated state. For example, a macrophage to achieve phagocytic activities requires a dynamic microfilament system while an epithelial cell requires the formation of highly organized microvilli to achieve optimal absorptive capacity. Further, the microfilaments through interactions with the plasma membrane is capable of modulating cellular responses to extracellular signals and also interactions with the substratum. Therefore the state of microfilament organization may influence the response of the cell to signals that might be expected to induce either proliferation or differentiation.

In conclusion, it is clear that the microfilament system is of vital importance to normal cellular functioning and further study of this system will lead to improved understanding not only of malignant transformation but also basic cell biology.

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ADDENDUM

References to the numbered points in this addendum have been inserted in the text.

1. Polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE), for almost all of the gels included in this thesis, was carried out using gels formed from 12% (w/v) acrylamide and 0.48% (w/v) DATD (N, N' Diallytartardiamide, from BIO-RAD Laboratories, Richmond, California). Gels were run using a Tris/glycine buffer (0.05M Tris, 0.39M glycine, pH 8.3, and 0.1% (w/v) SDS) for 4 hours at 300V or until the marker bromophenol blue reached the bottom of the gel. Samples were prepared for electrophoresis as detailed on page 153 of the thesis.

It should be noted that the crosslinking agent used to form the gels is DATD, in comparison to many gel systems where bis-acrylamide is the crosslinking agent. The use of differing percentage gels, different crosslinking agents, gels of differing lengths and running times can all influence the mobility of different proteins in the gel system. This can be seen from the gels included in this thesis where the mobility of actin relative to the 45,000 M_r marker (ovalbumin) varies slightly from one gel to another. In 9% bis-acrylamide gels, in the presence of SDS, highly purified actin runs slightly faster than ovalbumin while in a 10% bis-acrylamide gel this difference is marginal.

Because of this potential variation gels were always run with molecular weight markers and purified rabbit skeletal muscle actin included as references. When apparent molecular weights are given in the text they have been calculated using a calibration curve for the appropriate gel. Such calibration curves are constructed by plotting the logs of the molecular weights of the marker proteins against their relative mobilities, for example the calibration curve for figure 19b is attached. It should be noted that the calibration curves for the gel system used are not linear and accuracy is reduced for calculating apparent molecular weights less than 20,000 M_r or greater than 100,000 M_r. The calibration curve for figure 19b also indicates the identities of the marker proteins used (High and Low Molecular Weight Protein Standards for SDS Gel Electrophoresis from BIO-RAD Laboratories).

2. Composition of the Triton-Insoluble Pellet.

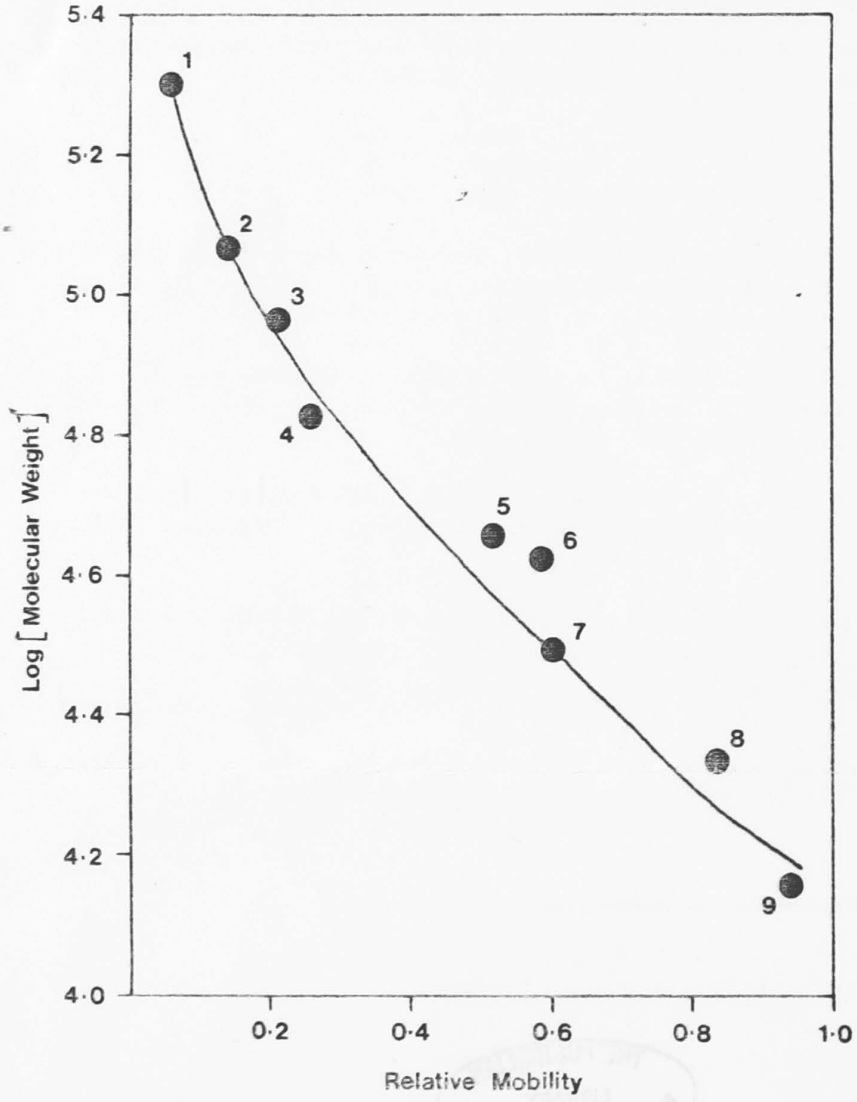
It is widely known that tubulin polymers tend to be depolymerised by cold conditions such as those used during the preparation of the triton-insoluble pellet. Hence it might be expected that tubulin would be present in monomeric form and would therefore be unlikely to be precipitated into the pellet. However, actin polymer also tends to be depolymerized by cold conditions and the observation that not all of the actin is depolymerized raises the possibility that the same may hold true for tubulin polymers. In addition, the triton-insoluble pellet is complex and tubulin may be precipitated as a result of interactions with other components of the pellet, such as intermediate filament proteins. Therefore, although the cold conditions should help to reduce the amount of tubulin present in the triton-insoluble pellet it must be considered to be a potential, if minor, component.

3. Actin severing/capping activity in the tumorigenic and nontumorigenic cell extracts.

The data shown in figure 20 suggests that an actin-binding protein activity, probably a barbed-end capping activity, may be present in greater amounts in the 0.6M NaCl wash from 5L (tumorigenic) cells than the 0.6M NaCl wash from the 5E (nontumorigenic) cells. The initial rate of actin polymerisation in the presence of the 0.6M NaCl wash from 5L cells is 0.45 units/min while the rate of actin polymerisation in the presence of the 0.6M NaCl wash from 5E cells is 2.12 units/min, a 4-fold difference in activity. This would appear to be a significant difference in specific activity given that the concentration of sample protein used should have been the same. However, because of the technical problems described in the text, the determination of protein content is unreliable. The degree of inaccuracy in the protein determination could well be sufficient to account for the 4-fold difference in activity in the 5E and 5L cell extracts. It should be emphasized that, while it remains possible that there is a difference in the level of specific actin-binding protein activity, because of the inaccuracy of protein determinations such a conclusion would be highly speculative. As indicated on p178, the yield and purity of the protein(s) of interest must be improved to allow measurement of specific actin-binding protein activity in tumorigenic and nontumorigenic cells to be compared with a greater degree of confidence.

Therefore, while it can be speculated that there could be increased amounts of F-actin capping/severing activities in the tumorigenic cells, the experimental data provided in the thesis cannot be used a supportive evidence for this hypothesis.

Calibration Curve for Figure 19b



KEY

PROTEIN	SUBUNIT MOLECULAR WEIGHT
1. Myosin	200,000
2. ß-Galactosidase	116,250
3. Phosphorylase B	92,500
4. Bovine Serum Albumin	66,200
5. Ovalbumin	45,000
6. Rabbit Skeletal Muscle Actin	42,000
7. Carbonic Anhydrase	31,000
8. Soybean Trypsin Inhibitor	21,500
9. Lysozyme	14,400